Chidambaram, JD; Prajna, NV; Palepu, S; Lanjewar, S; Shah, M; Elakkiya, S; Macleod, D; Lalitha, P; Burton, MJ (2018) In vivo confocal microscopy cellular features of host and organism in bacterial, fungal and Acanthamoeba keratitis. American journal of ophthalmology. ISSN 0002-9394 DOI: https://doi.org/10.1016/j.ajo.2018.03.010

Downloaded from: http://researchonline.lshtm.ac.uk/4647057/

DOI: 10.1016/j.ajo.2018.03.010

Usage Guidelines

Please refer to usage guidelines at http://researchonline.lshtm.ac.uk/policies.html or alternatively contact researchonline@lshtm.ac.uk.

Available under license: http://creativecommons.org/licenses/by/2.5/
In Vivo Confocal Microscopy Cellular Features of Host and Organism in Bacterial, Fungal, and Acanthamoeba Keratitis

JAYA D. CHIDAMBARAM, NAMPERUMALSAMY V. PRAJNA, SRIKANTHI PALEPU, SHRUTI LANJEWAR, MANISHA SHAH, SHANMUGAM ELAKKIYA, DAVID MACLEOD, PRAJNA LALITHA, AND MATTHEW J. BURTON

• PURPOSE: To determine cellular features of fungal (FK), Acanthamoeba (AK), and bacterial keratitis (BK) using HRT3 in vivo confocal microscopy (IVCM).
• DESIGN: Prospective observational cross-sectional study.
• METHODS: Eligible participants were adults with microbiologically positive FK, AK, or BK, of size ≥3 mm, attending Aravind Eye Hospital from February 2012 to February 2013. Exclusion criteria were descemetocele or perforation. At presentation, IVCM imaging was performed, then corneal scrapes were obtained for culture/light microscopy. An experienced grader (masked to microbiology/clinical features) assessed IVCM images for presence/absence of normal keratocyte-like morphology, stellate interconnected cells with/without visible nuclei, dendritiform cells (DFCs), inflammatory cells in a honeycomb distribution, and organism features. Statistical significance was assessed by logistic regression, adjusted for age, sex, ulcer size, and symptom duration. Main outcome measures were presence/absence of IVCM features in FK, AK, BK.
• RESULTS: A total of 183 participants had FK, 18 AK, 17 BK. Acanthamoeba appeared as bright spots (16/18, 89%), double-walled cysts (15/18, 83%), or signet rings (3/18, 17%), and often formed clusters after topical steroid use (univariable odds ratio [OR] 9.98, 95% confidence interval [CI] 1.02-97.96, P = .048). BK was associated with bullae in anterior stroma (OR 9.99, 95% CI: 3.11-32.06, P < .001). Honeycomb distribution of anterior stromal inflammatory cells was associated with FK (univariable OR 2.74, 95% CI: 1.01-7.40, P = .047). Aspergillus ulcers were associated with stromal DFCs (OR 11.05, 95% CI: 1.49-82.13, P = .019) and Fusarium ulcers with stellate appearance of interconnected cell processes with nuclei (OR 0.24, 95% CI: 0.09-0.65, P = .005).
• CONCLUSION: Specific cellular and structural features observed using IVCM in microbial keratitis may be associated with organism. (Am J Ophthalmol 2018;190:24–33. © 2018 The Author(s). Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).)
looked at the cellular changes in the cornea observed using IVCM in human MK.\textsuperscript{7,8,13} Since the molecular changes that occur in human MK caused by different organisms are subtly different,\textsuperscript{14} we postulated that the cellular changes in the cornea during infection may be different enough to allow use of these features to predict the infecting organism, even when the organism itself may not be apparent in the scan. In a prospective cohort of patients with moderate-to-severe bacterial keratitis (BK), Acanthamoeba keratitis (AK), and fungal keratitis (FK) in South India, we determined the IVCM appearance of the cornea at the cellular level. In addition, we documented specific features of organisms themselves that could be detected with this imaging modality, such as the “bright spot,” “doubled-walled cyst,” or “signet ring” appearance of Acanthamoeba cysts\textsuperscript{15,16}; spore-like structures along fungal filaments,\textsuperscript{17} or fine-beaded filamentous appearance of Nocardia sp.\textsuperscript{18,19}

**METHODS**

This study was prospectively approved by the Ethics Committees of the Indian Council for Medical Research, Aravind Eye Hospital, Tamil Nadu, India, and the London School of Hygiene and Tropical Medicine. As previously described, all patients gave written informed consent before enrolment; illiterate participants gave informed consent with a witnessed thumbprint on the study consent form (as approved by the above Ethics Committees).\textsuperscript{7} The tenets of the Declaration of Helsinki were adhered to during conduct of this study.

From February 28, 2012 to February 28, 2013, consecutive patients presenting to the Cornea Clinic of Aravind Eye Hospital, Madurai, Tamil Nadu, India were assessed for eligibility with the following inclusion criteria: age ≥ 18 years, stromal infiltrate diameter ≥ 3 mm, presence of overlying epithelial defect, and signs of acute inflammation. Patients were excluded if the ulcer had a descemetocele or epithelial defect, and signs of acute inflammation. Patients included in the grading were presence/absence of "bullae"—large, rounded areas of corneal thinning as assessed by slit-lamp examination (since application for IVCM could not safely be done in these patients), prior history of herpetic keratitis, or Snellen visual acuity worse than 6/60 in the unaffected eye, or if microbiologically negative (ie, culture and light microscopy) and IVCM-negative for any organism. At enrollment, data from a focused clinical history and slit-lamp examination were recorded. The cornea specialist examined every study participant and management was as per standard of care for microbial keratitis at Aravind Eye Hospital.

- **IN VIVO CONFOCAL MICROSCOPY IMAGING:** IVCM imaging of the corneal ulcer was performed using the HRT3 with Rostock Corneal Module (Heidelberg Engineering, Heidelberg, Germany) immediately prior to corneal scraping for microbiological tests, as described in detail elsewhere.\textsuperscript{10} Proparacaine 0.5% eye drop anesthesia was used (Aurocaine; Aurolab, Madurai, India) and the Rostock corneal module (Heidelberg Engineering) with 63× objective lens (Nikon, Tokyo, Japan) was gently applied to the corneal surface. The HRT3 IVCM was used in volume scan mode, which consists of a z-stack of 40 images covering a total of 80 μm corneal depth, each image with optical slice thickness of 2 μm. Images were obtained at the center of the ulcer and ulcer margins (12-, 3-, 6-, and 9-o’clock positions) with repeated volume scans performed to image the full depth of the cornea at each location, where possible.

After IVCM imaging, corneal scrapes were obtained from the leading margin and base of the ulcer to identify the causative organism via culture and light microscopy, using standard procedures described in detail elsewhere.\textsuperscript{7} For culture- and light microscopy–negative ulcers, 5 experienced IVCM graders assessed the IVCM images to determine the presence/absence of fungal hyphae or Acanthamoeba cysts, as described in our previous report.\textsuperscript{7}

- **IN VIVO CONFOCAL MICROSCOPY GRADING:** IVCM images were assigned a random identification number and were shuffled into a random order after removal of patient-identifying data. A single experienced grader performed all image grading and was masked to the clinical features and microbiological diagnosis. All data were recorded directly into a Microsoft Access database. The grading scheme included presence/absence of fungal hyphae, including presence/absence of spore-like structures (Figure 1), Acanthamoeba cyst features (double-wall, bright spot, signet ring, line, or cluster formation of cysts; Figure 1) or Nocardia sp. beaded filaments (Figure 1). Corneal stromal cellular appearances were graded as presence/absence of the following (as shown in Figures 2 and 3): “normal keratocyte-like morphology”—bright ovoid nuclei with barely visible processes (Figure 2, Top left; Figure 3, Left); “stellate cellular processes”—bright ovoid nuclei in a honeycomb formation either with bright ovoid nuclei (Figure 2, Top middle; Figure 3, Middle) or without nuclei (Figure 2, Top right; Figure 3, Right); "spindles”—linear bright nonbranching structures, often multiple and parallel to each other (Figure 2, Bottom left); and “granules”—small white opacities approximately 1-2 μm in diameter and present within the cells, either within the nuclear region or within the cellular processes (Figure 1, Middle). Other features included in the grading were presence/absence of “bullae” in the stroma or epithelium (Figure 2, Bottom right). The presence/absence of inflammatory cell appearances were also graded (Figure 4): “inflammatory cells in a honeycomb distribution”—bright round cells in alignment in a honeycomb contour (Figure 4, Left); “inflammatory cell infiltrate in a nonspecific distribution,” where a probable inflammatory cell infiltrate was detected but no
honeycomb distribution was observed (Figure 4, Right), or “dendritiform cells,” either in the basal epithelial layer (basal DFCs; Figure 4, Middle) or in the stroma (stromal DFCs). Images from the anterior half of the cornea (0-250 μm as measured using the IVCM pachymeter) or the posterior half (>250 μm) were graded separately. Acellular regions with homogenous reflectivity were classified as “scar” tissue.

FIGURE 1. In vivo confocal microscopy (IVCM) images showing: (Left) spore-like structures along fungal filaments; (Middle) Acanthamoeba cysts forming lines and clusters (with presence of granules within the stellate interconnected cellular processes shown by arrow); and (Right) fine beaded filamentous appearance of Nocardia sp. (indicated by arrows). Each IVCM image measures 400 × 400 μm.

FIGURE 2. In vivo confocal microscopy images. (Top left) Normal keratocyte-like morphology (bright ovoid nuclei, barely visible cellular processes); (Top middle) “stellate intercellular connectivity with nuclei visible” (bright ovoid nuclei and broad bright cellular processes interconnected in a honeycomb network); (Top right) “stellate intercellular connectivity with lack of visible nuclei.” (Bottom left) Linear “spindles” (arrow); (Bottom middle) bullae in stroma (arrow); (Bottom right) bullae in epithelium (arrow showing epithelial cell nucleus).
All statistical analyses were performed in Stata 12.1 (StataCorp, College Station, Texas, USA). We used standardized ordinal grading scales (ie, cellular feature present/absent), as has been previously used in ophthalmic histopathology studies of microbial keratitis. Baseline demographic features and IVCM features were compared between BK, AK, FK, and microbologically negative groups using \( \chi^2 \) test for proportions and Kruskal-Wallis test for continuous variables with nonparametric distribution. Logistic regression analysis was performed with the dependent variable as BK, AK, or FK vs all other ulcers combined, and with IVCM features as the independent variables. Initial analyses were performed with individual IVCM feature variables (adjusted for age, sex, ulcer size, and symptom duration), then the final multivariable analyses were also adjusted for any exposure (IVCM feature) where \( P < .1 \) in the initial analysis. Separate logistic regression analyses were performed for anterior and posterior corneal variables. Post hoc sub-group analysis was performed to compare IVCM features in Aspergillus keratitis to Fusarium keratitis to identify any differences in IVCM features between ulcers caused by these 2 fungi, which are the commonest causes of FK in the study cohort. All regression analyses were adjusted for age, sex, symptom duration, and ulcer stromal infiltrate size (defined as the geometric mean of longest stromal infiltrate diameter and its perpendicular diameter). Owing to collinearity, we only used ulcer stromal infiltrate size as a marker of disease severity, and did not include other signs (eg, ulcer depth, epithelial defect size).

RESULTS

OF THE 239 PARTICIPANTS ENROLLED IN THE STUDY, 17 WERE excluded owing to being microbologically negative (ie, no organism detected on culture, light microscopy, or IVCM).
and 4 were excluded owing to mixed bacterial/fungal infection (culture-positive for bacteria and positive for fungal hyphae on light microscopy and/or IVCM). Of the remaining 218 participants, 183 were diagnosed with fungal keratitis, 18 with Acanthamoeba keratitis, and 17 with bacterial keratitis, as summarized in Table 1. The baseline demographic profile of participants within each group (BK, AK, FK) showed no statistically significant differences in age, sex, presenting visual acuity, or proportion of ulcers with deep involvement of the posterior cornea (Table 2). However, in the AK group the symptom duration (median 30 days, \(P < .001\)) and ulcer size (median 6.8 mm diameter, \(P < .001\)) were greater than for all other ulcers. A total of 3153 volume scans consisting of 126 120 images were obtained at the enrollment visit in all patients (median 12 volume scans per patient, interquartile range 9-16). We were able to perform IVCM imaging of the posterior half of the cornea in 57 ulcers (Table 3), the majority of which were fungal (81%, 46/57), and culture-positive for Fusarium sp. (n = 22).

**IN VIVO CONFOCAL MICROSCOPY CELLULAR CHANGES IN FUNGAL KERATITIS:** The IVCM feature that occurred most frequently in the anterior stroma in FK compared to all other ulcers was the presence of inflammatory cells in a honeycomb distribution, found in 49% of FK (90/183) compared to 20% of all nonfungal ulcers (7/35, \(P = .001\), Table 3). In the logistic regression analysis, stromal bullae were independently associated with nonfungal rather than fungal ulcers (odds ratio [OR] 0.31, 95% confidence interval [CI] 0.11-0.82, \(P = .018\), Table 4). A honeycomb distribution of inflammatory cells in the absence of stromal bullae was more strongly associated with FK (OR 3.31, 95% CI 1.02-10.77, \(P = .046\)) than in the presence of stromal bullae (OR 0.47, 95% CI: 0.15-1.45, \(P = .189\)). In the multivariable analysis the evidence of an association between fungal ulcers and inflammatory cells in a honeycomb distribution disappeared upon inclusion of the stromal bullae variable, and so the former was not included in the final multivariable model. In the posterior cornea, there were no specific features that were associated with FK.

On comparison of Aspergillus keratitis (n = 33) with Fusarium keratitis (n = 73), ulcers with anterior stromal dendritiform cells had over 10 times the odds of being an Aspergillus ulcer than without (multivariable OR 11.05, 95% CI: 1.49-82.13, \(P = .019\)). However those ulcers with a stellate cellular appearance with visible nuclei present were associated with having one quarter of the odds of being Fusarium ulcers compared to those without (multivariable OR 0.24, 95% CI: 0.09-0.65, \(P = .005\)). No posterior stromal features were associated with Aspergillus or Fusarium ulcers.

With regard to fungal features, we observed spore-like structures in the anterior stromal IVCM images of 6 of the 183 FK cases (3%; Figure 1). Three of these cases were culture-positive for dematiaceous fungi (Curvularia sp. n = 1, Exserohilum sp. n = 1, unidentified dematiaceous fungus n = 1), 1 was culture-positive for Aspergillus flavus, and the remainder were culture-negative but light microscopy–positive for fungal filaments (n = 2). The median symptom duration for these 6 cases was 10 days (interquartile range, [IQR] 7-15 days), and the median stromal infiltrate size was 3.9 mm in diameter (IQR 3.2-9.7 mm).

**ACANTHAMOEBA KERATITIS:** Acanthamoeba ulcers were less likely to have a normal keratocyte-like morphology in the anterior stroma compared to all other causes of MK (multivariable OR 0.21, 95% CI: 0.06-0.79, \(P = .022\), Table 4). In the posterior stroma multivariable analysis, there was a lower strength of association for either appearance of stellate cellular processes with nuclei in non-AK ulcers (OR 0.03, 95% CI: <0.01-1.09, \(P = .056\)) or presence of intracellular granules in AK ulcers (OR 49.57, 95% CI: 0.94-2604.52, \(P = .053\), Table 4).

Acanthamoeba cysts were observed mainly as highly reflective bright spots (16/18, 89%) or with a double-walled morphology (15/18, 83%), rather than the signet ring appearance (3/18, 17%); in 14 patients,

### Table 1. Causative Organisms Identified by Culture, Light Microscopy, and In Vivo Confocal Microscopy

<table>
<thead>
<tr>
<th>Organism</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungi (n = 183)</td>
<td></td>
</tr>
<tr>
<td>Culture-positive (n = 144)</td>
<td></td>
</tr>
<tr>
<td>Fusarium sp.</td>
<td>73 33.5%</td>
</tr>
<tr>
<td>Aspergillus sp.</td>
<td>33 15.1%</td>
</tr>
<tr>
<td>Curvularia sp.</td>
<td>5 2.3%</td>
</tr>
<tr>
<td>Exserohilum sp.</td>
<td>4 1.8%</td>
</tr>
<tr>
<td>Lasiodiplodia sp.</td>
<td>2 0.9%</td>
</tr>
<tr>
<td>Cylindrocarpon sp.</td>
<td>1 0.4%</td>
</tr>
<tr>
<td>Bipolaris sp.</td>
<td>1 0.4%</td>
</tr>
<tr>
<td>Unidentified hyaline fungi</td>
<td>14 6.4%</td>
</tr>
<tr>
<td>Unidentified dematiaceous fungi</td>
<td>11 5.0%</td>
</tr>
<tr>
<td>Culture-negative but light</td>
<td></td>
</tr>
<tr>
<td>microscopy-positive for fungi</td>
<td></td>
</tr>
<tr>
<td>Culture-negative but IVCM-positive for fungi</td>
<td>9 4.1%</td>
</tr>
<tr>
<td>Acanthamoeba (n = 18)</td>
<td></td>
</tr>
<tr>
<td>Culture-positive</td>
<td>17 7.8%</td>
</tr>
<tr>
<td>Culture-negative but IVCM-positive for Acanthamoeba</td>
<td>1 0.5%</td>
</tr>
<tr>
<td>Bacteria (n = 17)</td>
<td></td>
</tr>
<tr>
<td>Culture-positive (n = 17)</td>
<td></td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>9 4.1%</td>
</tr>
<tr>
<td>Nocardia sp.</td>
<td>3 1.4%</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>2 0.9%</td>
</tr>
<tr>
<td>Aeromonas sp.</td>
<td>1 0.4%</td>
</tr>
<tr>
<td>Streptococcus viridans</td>
<td>1 0.4%</td>
</tr>
<tr>
<td>Staphylococcus epidermidis</td>
<td>1 0.4%</td>
</tr>
<tr>
<td>Total</td>
<td>218 100%</td>
</tr>
</tbody>
</table>

IVCM = in vivo confocal microscopy.
both bright spot and double-wall cyst morphologies were present in the IVCM images. The cysts appeared to group together into lines (7/18, 39%) or clusters (6/18, 33%), as shown in Figure 1. Specifically, prior steroid use was more strongly associated with the formation of clusters (OR 9.98, 95% CI 1.02-97.96, \( P = .048 \)) rather than lines of cysts (OR 2.43, 95% CI: 0.36-16.48, \( P = .363 \); Table 4).

### Table 3. Baseline Characteristics of Study Participants

<table>
<thead>
<tr>
<th></th>
<th>Fungal Keratitis (77.4%, N = 183)</th>
<th>Acanthamoeba Keratitis (8.3%, N = 18)</th>
<th>Bacterial Keratitis (7.8%, N = 17)</th>
<th>( P ) Value$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age, years (IQR)</td>
<td>50 (36-58)</td>
<td>39 (34-55)</td>
<td>60 (46-65)</td>
<td>.104</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>118 (64.8%)</td>
<td>11 (61.1%)</td>
<td>10 (58.8%)</td>
<td>.871</td>
</tr>
<tr>
<td>Symptom duration, median number of days (IQR)</td>
<td>7 (4-10)</td>
<td>30 (7-60)</td>
<td>8 (4-14)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Baseline visual acuity, median logMAR (IQR)</td>
<td>1.8 (0.6-1.8)</td>
<td>1.8 (1.7-1.8)</td>
<td>1.7 (1.7-1.8)</td>
<td>.244</td>
</tr>
<tr>
<td>Ulcer stromal infiltrate size, ( \mu ) mm (median, IQR)</td>
<td>4.4 (3.3-5.5)</td>
<td>6.8 (5.3-8.0)</td>
<td>3.7 (3.2-5.0)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Deep infiltrate involving posterior one-third of cornea, n (%)</td>
<td>113 (62.1%)</td>
<td>13 (72.2%)</td>
<td>12 (70.6%)</td>
<td>.585</td>
</tr>
</tbody>
</table>

$^a$Ulcer stromal infiltrate size calculated as geometric mean of longest diameter and perpendicular diameter.

$^b$Differences between all 3 groups assessed for statistical significance using \( \chi^2 \) test for proportions (sex, ulcer depth) and Kruskal-Wallis test for continuous nonparametric variables.

### Table 2. Baseline Characteristics of Study Participants

<table>
<thead>
<tr>
<th></th>
<th>Fungal Keratitis (N = 183)</th>
<th>Acanthamoeba Keratitis (N = 18)</th>
<th>Bacterial Keratitis (N = 17)</th>
<th>( P ) Value$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age, years (IQR)</td>
<td>50 (36-58)</td>
<td>39 (34-55)</td>
<td>60 (46-65)</td>
<td>.104</td>
</tr>
<tr>
<td>Male sex, n (%)</td>
<td>118 (64.8%)</td>
<td>11 (61.1%)</td>
<td>10 (58.8%)</td>
<td>.871</td>
</tr>
<tr>
<td>Symptom duration, median number of days (IQR)</td>
<td>7 (4-10)</td>
<td>30 (7-60)</td>
<td>8 (4-14)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Baseline visual acuity, median logMAR (IQR)</td>
<td>1.8 (0.6-1.8)</td>
<td>1.8 (1.7-1.8)</td>
<td>1.7 (1.7-1.8)</td>
<td>.244</td>
</tr>
<tr>
<td>Ulcer stromal infiltrate size, ( \mu ) mm (median, IQR)</td>
<td>4.4 (3.3-5.5)</td>
<td>6.8 (5.3-8.0)</td>
<td>3.7 (3.2-5.0)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Deep infiltrate involving posterior one-third of cornea, n (%)</td>
<td>113 (62.1%)</td>
<td>13 (72.2%)</td>
<td>12 (70.6%)</td>
<td>.585</td>
</tr>
</tbody>
</table>

$^a$Ulcer stromal infiltrate size calculated as geometric mean of longest diameter and perpendicular diameter.

$^b$Differences between all 3 groups assessed for statistical significance using \( \chi^2 \) test for proportions (sex, ulcer depth) and Kruskal-Wallis test for continuous nonparametric variables.

### Table 3. Cellular Features Detected Within In Vivo Confocal Microscopy Images of Bacterial, Fungal, Acanthamoeba, and Microbiologically Negative Keratitis

<table>
<thead>
<tr>
<th>Corneal Location</th>
<th>IVCM Features</th>
<th>Fungal Keratitis (N = 183)</th>
<th>Acanthamoeba Keratitis (N = 18)</th>
<th>Bacterial Keratitis (N = 17)</th>
<th>( P ) Value$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior</td>
<td>Normal keratocyte-like morphology</td>
<td>141 (77%)</td>
<td>7 (39%)</td>
<td>14 (82%)</td>
<td>.001</td>
</tr>
<tr>
<td></td>
<td>Stellate cellular processes with nuclei</td>
<td>119 (65%)</td>
<td>6 (33%)</td>
<td>11 (65%)</td>
<td>.029</td>
</tr>
<tr>
<td></td>
<td>Stellate cellular processes no nuclei</td>
<td>58 (32%)</td>
<td>10 (56%)</td>
<td>3 (18%)</td>
<td>.047</td>
</tr>
<tr>
<td></td>
<td>Spindles</td>
<td>132 (72%)</td>
<td>14 (76%)</td>
<td>13 (76%)</td>
<td>.826</td>
</tr>
<tr>
<td></td>
<td>Granules</td>
<td>108 (59%)</td>
<td>13 (72%)</td>
<td>11 (65%)</td>
<td>.514</td>
</tr>
<tr>
<td></td>
<td>Epithelial bullae</td>
<td>18 (10%)</td>
<td>0 (0%)</td>
<td>7 (41%)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td></td>
<td>Stromal bullae</td>
<td>19 (10%)</td>
<td>1 (6%)</td>
<td>8 (47%)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td></td>
<td>Inflammatory cells (honeycomb)</td>
<td>90 (49%)</td>
<td>1 (6%)</td>
<td>6 (35%)</td>
<td>.001</td>
</tr>
<tr>
<td></td>
<td>Inflammatory cells (nonspecific)</td>
<td>42 (23%)</td>
<td>3 (17%)</td>
<td>6 (35%)</td>
<td>.403</td>
</tr>
<tr>
<td></td>
<td>Basal DFCs</td>
<td>97 (53%)</td>
<td>7 (39%)</td>
<td>14 (82%)</td>
<td>.027</td>
</tr>
<tr>
<td></td>
<td>Stromal DFCs</td>
<td>19 (10%)</td>
<td>5 (28%)</td>
<td>4 (23%)</td>
<td>.043</td>
</tr>
<tr>
<td></td>
<td>Scar</td>
<td>19 (10%)</td>
<td>4 (22%)</td>
<td>3 (18%)</td>
<td>.251</td>
</tr>
<tr>
<td></td>
<td>Fungal spore-like structures</td>
<td>6 (3%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Corneal Location</th>
<th>IVCM Features</th>
<th>Fungal Keratitis (N = 46)</th>
<th>Acanthamoeba Keratitis (N = 7)</th>
<th>Bacterial Keratitis (N = 4)</th>
<th>( P ) Value$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Posterior</td>
<td>Normal keratocyte-like morphology</td>
<td>31 (67%)</td>
<td>4 (57%)</td>
<td>4 (100%)</td>
<td>.320</td>
</tr>
<tr>
<td></td>
<td>Stellate cellular processes with nuclei</td>
<td>35 (76%)</td>
<td>2 (29%)</td>
<td>3 (75%)</td>
<td>.037</td>
</tr>
<tr>
<td></td>
<td>Stellate cellular processes no nuclei</td>
<td>10 (22%)</td>
<td>5 (71%)</td>
<td>1 (25%)</td>
<td>.024</td>
</tr>
<tr>
<td></td>
<td>Spindles</td>
<td>30 (65%)</td>
<td>6 (86%)</td>
<td>2 (50%)</td>
<td>.430</td>
</tr>
<tr>
<td></td>
<td>Granules</td>
<td>22 (48%)</td>
<td>6 (86%)</td>
<td>2 (50%)</td>
<td>.173</td>
</tr>
<tr>
<td></td>
<td>Inflammatory cells (honeycomb)</td>
<td>11 (24%)</td>
<td>1 (14%)</td>
<td>0 (0%)</td>
<td>.476</td>
</tr>
<tr>
<td></td>
<td>Inflammatory cells (nonspecific)</td>
<td>8 (17%)</td>
<td>0 (0%)</td>
<td>2 (50%)</td>
<td>.111</td>
</tr>
<tr>
<td></td>
<td>Stromal DFCs</td>
<td>2 (4%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>.780</td>
</tr>
<tr>
<td></td>
<td>Scar</td>
<td>0 (0%)</td>
<td>1 (14%)</td>
<td>0 (0%)</td>
<td>.026</td>
</tr>
<tr>
<td></td>
<td>Fungal spore-like structures</td>
<td>2 (4%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>-</td>
</tr>
</tbody>
</table>

DFCs = dendritiform cells; IVCM = in vivo confocal microscopy.

$^a$Statistical significance of difference between all 3 groups assessed using \( \chi^2 \) test.
BACTERIAL KERATITIS: Epithelial and anterior stromal bullae were the main features that were associated with bacterial keratitis in the univariable analysis compared to all other causes of MK (OR 5.72, 95% CI: 1.73-18.94, P = .004, and OR 9.99, 95% CI: 3.11-32.06, P < .001, respectively; Table 4). Stromal bullae alone remained strongly associated with BK in multivariable analysis; the reduction in strength of evidence to support association between BK and epithelial bullae when included with stromal bullae in the multivariable model may be attributable to the independent association of epithelial bullae with bacterial ulcers and also with stromal bullae (ie, most likely along the causal pathway). DFCs in the basal epithelial layer (univariable OR 3.74, 95% CI: 1.00-13.91, P = .49) and in the anterior stroma (univariable OR 3.51, 95% CI: 0.88-14.09, P = .076) were very weakly associated with bacterial keratitis rather than any other cause of MK, but this did not reach statistical significance in the multivariable model.

There were 3 ulcers that were culture-positive for Nocardia sp. We only observed possible Nocardia filaments in 1 of these 3 ulcers (Figure 1). The grader also recorded the presence of Nocardia-like fine, beaded filaments in 1 other ulcer, which was culture-positive for an unidentified dematiaceous fungus.

**DISCUSSION**

*Here we have described the cellular changes that occur in the cornea in MK as observed with IVCM at first presentation. In FK, which formed the majority of cases in this study of large ulcers, the only IVCM feature weakly associated with this disease was the presence of an anterior stromal honeycomb distribution of inflammatory cells. This specific honeycomb pattern of inflammatory cells is similar to that observed after abrasion injury in real-time in vivo HRT3 IVCM imaging of the mouse cornea; these inflammatory cells were identified as neutrophils using immunohistochemistry in the same tissue ex vivo, and their close interaction with keratocytes was found to be mediated by...*
through action of cell adhesion molecules. However, to
our knowledge, this honeycomb distribution of migrating
inflammatory cells has not been formally investigated in
FK before. Neutrophils are recruited to the cornea very
soon after the onset of infection in MK, even within hours,
and this is mediated through release of chemokines in the
cornea by host cells (eg, CXCL1, CXCL5, IL8).14,21

The nature of the corneal cellular response to fungal
infection may also differ in Aspergillus vs Fusarium keratitis;
in the IVCM images of the anterior corneal stroma, we
observed associations between dendritiform cells and
Aspergillus ulcers in our subgroup analysis. Since Aspergillus
keratitis is often more difficult to treat, with greater risk of
poor outcomes, larger studies are needed to more fully
ascertain whether there may be IVCM cellular features
that are associated with this fungus to aid diagnosis and
management of these cases.22

In 6 of the FK cases, we detected fungal spore-like struc-
tures that were present along hyphae in the anterior stroma.
These most likely represent chlamydospores, which are
thick-walled structures along hyphae that typically occur
in fungi that have depleted their local nutrient supply.23
Chlamydospores have been previously reported in corneal
scrapings from human FK, predominantly in ulcers that
were culture-positive for dematiaceous fungi such as Curvu-
laria sp.17 In our study, 3 of the 6 ulcers with spore-like
structures detected on IVCM were culture-positive for
dematiaceous fungi. Others have shown that the presence
of fungal spores within tissues is frequently associated
with disseminated disease and poor prognosis.24 Similarly
in FK, the appearance of spore-like structures within
IVCM images may be an indicator of worsening of disease,
and so further studies are required to elucidate its prog-
nostic value.

In AK, the main IVCM features associated were a lack of
normal keratocyte-like morphology in the anterior stroma
compared to the other causes of MK. Acanthamoeba are
able to kill keratocytes through other mechanisms such as
direct cytopathic effects, phagocytosis, and induction of
apoptosis or necrosis, as shown in both in vitro studies
and histologic studies, although apoptosis is most likely
to be the predominant method by which keratocyte death
occurs in AK.25,26 Although we were not able to perform
immunohistochemical studies to confirm apoptosis,
others have done so and found through the use of
TUNEL staining that apoptosis of keratocytes does
indeed occur throughout the corneal stroma in human
AK, BK, and FK, and particularly in the posterior stroma
in AK.20,26 We were only able to study a small number of
Acanthamoeba ulcers in this study, and so larger studies
are required to confirm these IVCM findings.

We also found that AK cyst-like structures formed clus-
ters, particularly in ulcers that had undergone treatment
with topical steroid prior to presentation. Yokogawa and
associates observed that clusters of Acanthamoeba cysts
in the Bowman membrane were present in cases of persistent
AK, and that a high proportion of these patients had used
topical steroid therapy prior to presentation.27 Zhang and
associates also noted that the formation of lines or clusters
of AK cysts was associated with poor prognosis in their se-
ries of 29 patients with AK, although steroid use was not
mentioned.28 Reasons for in vivo cluster formation of
Acanthamoeba cysts with or without steroid exposure
remain to be elucidated, but prior studies have shown
that Acanthamoeba are able to adhere to multiple surfaces,
including contact lenses,29 corneal extracellular matrix
components (eg, collagens and laminins),30 and host
corneal epithelial cells.31 Exposure of Acanthamoeba cysts
to dexamethasone increases their cytopathic effect on
host corneal cells, and this could be one reason for poor
prognosis in patients treated with topical steroid alone.32
Larger studies are needed to identify whether the formation
of clusters of Acanthamoeba cysts in IVCM images is a useful
prognostic indicator in AK.

DFCs in the IVCM images of ocular disease have been
used as a predictor of causative organism. Cruzat and asso-
ciates studied the presence of basal DFCs only (not stromal
DFCs) in HRT3 IVCM images of AK, BK and FK, and
found that AK had a higher density of DFCs in this region
of the cornea.1 We found that a higher proportion of IVCM
images from the BK group had basal DFCs, rather than in
AK or FK. The difference may be related to prior steroid
use, since many of the BK patients in the study by Cruzat
and associates had used topical steroids beforehand,
whereas only 1 BK patient in our study had a history of ste-
roid use. Multiple cell types can take on a dendritiform
morphology, as observed with confocal microscopy (both
IVCM and ex vivo). Corneal tissue–resident macrophages,
dendritic cells, and even keratocytes can possess this elon-
gated, dendritiform cell shape, in addition to bone marrow–
derived myeloid cells that have migrated into the inflamed
cornea.6,33–35 Future studies directly comparing IVCM
imaging with immunostaining of the same tissue ex vivo
would aid in identifying the cell of origin of the
morphologies that we have described in this report, and
would provide further information on the pathogenesis
of disease.

In contrast to AK and FK, the occurrence of bullae in the
epithelium and stroma were associated with BK. Epithelial
bullae have been observed in IVCM images of Fuchs endo-
thelial dystrophy in the past, and ascribed to tissue edema
causing microcysts within the epithelial layer.36 The larger
bullae seen within the corneal stroma may be an indication
of stromal tissue damage. Most of the BK cases in this
study were culture-positive for Streptococcus pneumoniae. This
organism contributes to host tissue damage through multiple
mechanisms, including release of reactive oxygen species
and excessive stimulation of host cells (eg, neutrophils) to
release matrix metalloproteinases that can also destroy host
tissue.37 Control of this damage through use of topical ste-
roid treatment early on in bacterial corneal ulceration may
have some impact on improving final visual outcome in
large ulcers that are in the visual axis.\textsuperscript{39} IVCM may be a useful tool for monitoring the effect of any treatment regime on the stromal necrotic response.

\textit{Nocardia} sp. have been documented to appear as thin beaded filamentous structures in IVCM images\textsuperscript{13,19,20} and are therefore one of the few bacterial causes of keratitis that can be visualized with this imaging modality.\textsuperscript{13} \textit{Nocardia} sp. filaments are smaller in diameter than \textit{Aspergillus} or \textit{Fusarium} fungal hyphae (up to 1 \textmu m compared to 3-6 \textmu m for filamentous fungi).\textsuperscript{11,20} We only observed thin beaded filaments in the IVCM images of 1 out of the 3 \textit{Nocardia} ulcers in our study, and also in 1 ulcer that was culture-positive for a dematiaceous fungus. As such, it may not always be possible to rely on direct visualization of thin beaded filaments in IVCM images to make the diagnosis of \textit{Nocardia} keratitis.

A limitation of our study is that we were able to enroll only a small number of bacterial and \textit{Acanthamoeba} ulcers. Since this was a prospective cohort study, the observation that the majority of our cases were fungal reflects the distribution of causative organisms of large ulcers in South India. Larger studies are needed in the future to more fully elucidate the IVCM features that we have reported for bacterial and \textit{Acanthamoeba} keratitis. Also, we chose to only enroll large ulcers, as we felt that these can often pose a greater diagnostic challenge and frequently have a worse visual outcome. Ulcers of a smaller size at presentation may have lesser tissue damage at presentation, and so different IVCM cellular findings, which need to be investigated in the future.

In summary, here we show that patterns of cellular changes as detected with IVCM may be helpful in predicting the causative organism in MK. In addition to diagnosing the pathogen, IVCM allows an insight into the histology of the living cornea during infection and the cellular host response. Future studies are required to explore the use of IVCM in particular for monitoring therapeutic response.

---

**REFERENCES**


