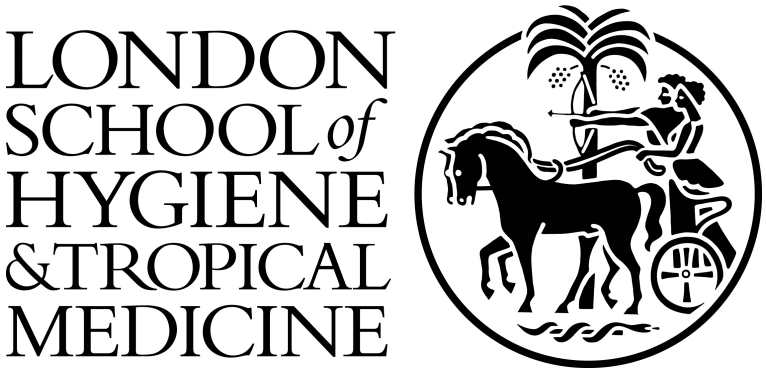

Studies in the Diagnosis and Pathophysiology of Severe Microbial Keratitis

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Declaration

I, Jaya Devi Chidambaram, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Signature: 

Date: 9th August, 2018

Abstract

Background: Microbial keratitis (MK) is a major cause of blindness worldwide. Few studies have assessed the HRT3 *in vivo* confocal microscope (IVCM) in the diagnosis or management of MK. This PhD aimed to investigate these questions and was based in a high incidence setting for MK: Aravind Eye Hospital, Madurai, India.

Methods: A prospective observational study of 252 severe MK patients was conducted with follow-up at days 7, 14 and 21 post-enrolment. Quantitative PCR of corneal swabs obtained at presentation validated transcriptome results. IVCM images were evaluated in this cohort for diagnostic accuracy, and ability to monitor outcome.

Results: For fungal detection, HRT3 IVCM had a high sensitivity (85.7%; 95% CI: 82.2%–88.6%) and specificity (81.4%; 95% CI: 76.0%–85.9%). For *Acanthamoeba*, the sensitivity was 88.2% (95% CI: 76.2%–94.6%) and specificity was 98.2% (95% CI: 94.9%–99.3%). Mean fungal branching angle in IVCM images was not significantly different for *Fusarium* sp. (59.7°; 95% CI: 57.7°–61.8°) versus *Aspergillus* sp. (63.3°; 95% CI: 60.8°–65.8°; p=0.07). At presentation, anterior corneal IVCM morphology associated with BK included bullae (OR 9.99, 95% CI: 3.11–32.06, p<0.001), and in FK a honeycomb distribution of inflammatory cells (OR 2.74, 95%CI: 1.01–7.40, p=0.047). Poor outcomes in FK were associated with stellate interconnected cellular processes with no visible nuclei (OR 2.28, 95% CI: 1.03-5.06, p=0.043) in baseline IVCM images, and fungal filaments (OR 6.48, 95% CI:2.50-16.78, p<0.001) or inflammatory cells in a honeycomb distribution (OR 5.24, 95% CI: 1.44-19.06, p=0.012) in final visit images.

Conclusions: HRT3 IVCM can yield a high diagnostic accuracy. Fungal branching angle in IVCM images does not differentiate between *Fusarium* and *Aspergillus* keratitis. IVCM image morphologies may be associated with causative organism or clinical outcome in MK.

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List of Abbreviations

AEH	Aravind Eye Hospital
AK	*Acanthamoeba* keratitis
BA	Blood agar
BK	Bacterial keratitis
C	Control tissue
CI	Confidence interval
CFW	Calcofluor white
DFC	Dendritiform cells
DM	Descemet's membrane
FK	Fungal keratitis
HRT	Heidelberg retinal tomograph
IVCM	*In vivo* confocal microscopy
KOH	10% potassium hydroxide
MK	Microbial keratitis
NPV	Negative predictive value
OCT	Optical coherence tomography
OR	Odds ratio
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PPV	Positive predictive value
PRK	Photo-refractive keratectomy
RCM	Rostock corneal module
STARD	Standards for reporting of diagnostic accuracy studies
TSCM	Tandem scanning confocal microscope

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Contributors to the Research Presented in this Thesis

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Chapter 1

Introduction

Study patient having slit lamp biomicroscopy at first study visit

1.1 Microbial Keratitis (MK) in India: Burden of disease

Corneal ulceration due to an infective cause, also known as microbial keratitis (MK), is a major cause of blindness worldwide.[1] Prospective studies have shown a 2.8-fold higher incidence of MK in India (113 per 100, 000 persons)[2], compared to the UK (40 per 100,000 persons).[3] The World Health Organisation reported in the most recent global blindness data that corneal opacity caused approximately 4% of all global blindness and 1% of all visual impairment worldwide. In South India, the incidence of MK is approximately 1700 patients per year[4] in Madurai district (one of the largest districts in Tamil Nadu), and it has been estimated that 1 in 150 people in the South Indian population currently have corneal blindness in one or both eyes, mostly as a result of MK.[5] Extrapolating to the estimated population of India in the year 2020, this equates to approximately 10.6 million people with unilateral corneal blindness by that time.[5]

MK is mainly caused by bacteria, fungi or *Acanthamoeba* sp. In India, due the wide variation in climate and humidity from North to South, there is a marked difference in the spectrum of causative organisms in each region.[6] In the warmer, more humid parts of South India such as Tamil Nadu, filamentous fungi such as *Fusarium* sp. predominate,[4] whereas *Aspergillus* sp. and *Candida* sp. are more frequent pathogens in cooler climates of North India.[7] The main bacterial pathogen for MK in India is most frequently either *Staphylococcus* sp. or *Streptococcus pneumoniae*, with *Pseudomonas* sp. occurring less frequently.[4, 8, 9]

One of the key challenges at present in the management of MK is the difficulty in using slit lamp examination alone to determine the causative organism or to assess response to treatment. Early diagnosis of the correct pathogen, and therefore the ability to start the correct antimicrobial can result in better visual outcomes, however based upon clinical features alone this can be difficult, even for experienced clinicians.[10] Classically, features associated with fungal ulcers are feathery margins and satellite lesions, and with *Acanthamoeba* ulcers are ring infiltrates, but ulcers may not always present with such a clinical picture.[11, 12] Although microbiological methods such as culture and light microscopy of corneal scrapes therefore remain the reference standard for identifying the causative organism in MK, in some settings over 60% of corneal scrapes are microbiologically-negative.[8] Use of high resolution imaging such as the HRT3 laser scanning in vivo confocal microscopy gives a cellular view of the living cornea allowing rapid identification of pathogens such

as fungi and *Acanthamoeba* however very few studies have formally investigated the use of this modality in diagnosis or management of MK.

Finally, even after commencing the correct antimicrobial treatment, continued tissue destruction can ensue, resulting in significant central corneal scarring or thinning or perforation. The pathophysiology involved in these changes has been explored in various studies, including animal models, which have revealed the huge interplay between various biological pathways that can ultimately result in disease resolution or worsening. However, no study to date has explored the whole transcriptome in the human cornea in MK.

In this set of inter-related PhD studies, we aimed to assess the use of IVCM to diagnose and manage severe MK, as well as to explore the pathophysiology of human disease as viewed through transcriptomics. Since the increasing incidence of MK in India has been described as a "silent epidemic" and frequently causes visual impairment or blindness of those of in the working population,[13] we feel that these PhD studies will be of value in contributing towards greater knowledge of MK.

1.2 Microbial Keratitis (MK) in India: Epidemiology

The epidemiology of MK in India is strongly associated with the geographical region. Since the South Indian states of Tamil Nadu, Andhra Pradesh, Telangana, Kerala and Karnataka are closer to the equator, and have a much hotter climate with increased humidity, the climate appears to promote *Fusarium* corneal infection which requires a wet environment for spread of its spores.[14] In cooler climates, such as in North India, keratitis caused by *Aspergillus* sp. is more common, presumably due to the drier environment required for airborne spread of spores by this fungus.[14] Trauma to the eye from vegetative matter during agricultural work is a well known risk factor for MK and especially FK (see Table 1).[15, 16] Plant pathogens such as *Fusarium* sp. may be present in crops and therefore corneal trauma during harvesting allows a direct route of entry to initiate an episode of MK.[14] This is reflected in a seasonal peak of MK, in particular FK, that been found to occur around harvest season or the monsoon season in some regions of India.[7, 9, 15-24]

Table 1: Summary of the main epidemiological risk factors associated with clinically suspected microbial keratitis (MK) in India (including studies specific for bacterial keratitis, BK, fungal keratitis, FK, or Acanthamoeba keratitis, AK). NB: Table 2 contains data on the proportion of BK, FK and AK in each study, and specific organisms cultured.

***Search methodology: PubMed database (https://www.ncbi.nlm.nih.gov/pubmed/) accessed on 10 August 2017 using search terms "bacteria" or "fung*" or "myco*" or "acanthamoeba", combined with "epidemiology", or "incidence" or "prevalence", and "keratitis" or "corneal ulcer", and "India"; review articles excluded). Abbreviations: NM=not mentioned, M=male, F=female, CL=contact lens wear, DM=diabetes mellitus.**

India Zone	State & City	First author, publication year (study time period)	Male to Female ratio	Peak Age, years (%)	Agri-cultural work	Rural resid-ence	Trauma	Trauma with Vege-tative Matter	DM	Prior Topical Steroid Use	CL Wear	Other Risk Factors
North	New Delhi	Gupta A, 2014[25] (2004-12)	1.75M : 1F	21-40 (% NM)	Mostly agricultural workers (% NM)	NM	NM	48%	NM	25% with antibiotic	NM	-
North	Chandigarh, Haryana & Punjab	Punia RS, 2014[26] (2003-12)	1.2M : 1F (in FK)	41-60 (FK)	NM	NM	59% (26/44; in FK)	NM	NM	18% with antibiotic (8/44 in FK)	0	-
North	Ujjain, Madhya Pradesh	Sharma V, 2008[7] (2006-7)	M>F	21-50	19% (37)	NM	NM	17% (34)	NM	1% (2)	2% in FK (1/42)	Seasonal peak of FK after soybean harvest (Nov) or wheat (Mar)
North	Chandigarh, Haryana & Punjab	Chander J, 2008[27] (1999-2003)	4M :1F	21-50	69% (106/154)	77% (118/154)	44% in FK (28/64)	37% in FK (24/64)	NM	8% in FK (5/64)	NM	-
North	New Delhi	Chowdhary A, 2005[28] (1999-2001)	2.1M : 1F (FK only)	31-40 (36%; FK only)	NM	NM	NM	NM	NM	NM	NM	-

Table 1 continued

India Zone	State & City	First author, publication year (study time period)	Male to Female ratio	Peak Age, years (%)	Agri-cultural work	Rural resid-ence	Trauma	Trauma with Vege-tative Matter	DM	Prior Topical Steroid Use	CL Wear	Other Risk Factors
North	Srinagar, Jammu & Kashmir	Bashir G, 2005[29] (1999-2001	M>F	NM	61%	NM	NM	NM	NM	NM	NM	-
North	Chandigarh, Haryana & Punjab	Chander J, 1994[30] (1988-1994)	3M: 1F (FK)	51-60 (FK)	NM	Rural>Urban (in FK, %NM)	NM	NM	NM	NM	NM	-
South	Bangalore, Karnataka	Ranjini CY, 2016[8] (2012-14)	1.6M: 1F	41-60 (% NM)	NM	37.5% (n=117/312)	46% (n=54/117)	NM	26% (n=31/117)	3% (n=4/117)	19 (n=22/117)	-
South	Coimbatore, Tamil Nadu	Manikandan P, 2013[17] (2005-8)	1.5M: 1F (Aspergillus FK)	21-70 (Aspergillus FK; 88%, 176/200)	25% (50/176 Aspergillus FK)	NM	54% (108/200 Asp FK)	NM	NM	8.5% (17/200)	NM	Seasonal peak June-Sept (43% Aspergillus cases)
South	Hyderabad, Telangana	Gopinathan U, 2009[9] (1991-2001)	2.25M: 1F	Mean 41.2 yrs (± 20.4) in BK, 30.9 yrs (± 15.3) in FK, 34.4 yrs (± 12.5) in AK	24% all MK (827/3448; p<0.001)	NM	FK 82% (712/869; p<0.001); AK 96% (42/44p=0.02)	NM	69.2% (205/296)	10% all MK (196/1945); BK 171/196; FK 15/196	0.8% all MK (AK: 1/118)	Seasonal peak for FK and AK in monsoon

Table 1 continued

India Zone	State & City	First author, publication year (study time period)	Male to Female ratio	Peak Age, years (%)	Agri-cultural work	Rural resid-ence	Trauma	Trauma with Vege-tative Matter	DM	Prior Topical Steroid Use	CL Wear	Other Risk Factors
South	Tirunelveli, Tamil Nadu	Jayahar Bharathi M, 2007[15, 31] (1999-2002)	1.4M: 1F	21-50 all MK (52%); 21-50 FK (67%; p<0.0001); >50 yrs in BK (60%, p<0.0001)	65% FK (OR 1.4, 95% CI: 1.2-1.6, p<0.0001); 42% BK; 79% AK (p=0.031)	70% all MK (2229/3183); 80% FK (p<0.0001); 100% AK (p<0.0001)	71% all MK (2256/3183) 92% in FK (p<0.0001); 100% AK (33/33)	61% FK (1009/1095; OR 23.6, 95% CI: 19.1-29.3, p<0.0001)	7% (211/3183); 16% FK (172/1095); 3% BK (30/1043)	1% all MK (20/3183); 1% FK (13/1095)	1% all MK (33/3183) – all were BK	BK: 58% non-agriculturalists (OR 2.9, 95% CI: 2.5-3.4, p<0.0001). AK: Mud in eye in 85% (OR 26.0, 95% CI: 3.3-6.7, p<0.0001). FK seasonal peak June-Sept.
South	Coimbatore, Tamil Nadu	Manikandan P, 2004[32] (1997-2003)	1.7M: 1F (AK)	31-40 (AK)	56% (18/32)	NM	54% (18/33)	42% (14/33)	NM	NM		
South	Hyderabad, Telangana	Gopinathan U, 2002[18] (1991-2000)	2.5 M: 1F	16-49 (FK)	47% (642/1352 FK)	NM	54% (736/1352 FK)	14% (188/1352 FK)	6% (87/1352 FK)	3% antimicrobial +/- steroid (37/1352 FK)	NM	Seasonal peak of FK in June to Jan (monsoon and winter)
South	Hyderabad, Telangana	Sharma S, 2000[33] (1996-8)	1.4M: 1F	21-40 (AK) (25/39; 64%)	NM	NM	38% AK (15/39)	38% AK (15/39)	NM	NM	0%	Unclean water into eye 8% AK (3/39)

Table 1 continued

India Zone	State & City	First author, publication year (study time period)	Male to Female ratio	Peak Age, years (%)	Agri-cultural work	Rural resid-ence	Trauma	Trauma with Vege-tative Matter	DM	Prior Topical Steroid Use	CL Wear	Other Risk Factors
South	Madurai, Tamil Nadu	Srinivasan, M, 1997[13] (1994)	1.6M: 1F	31-60 (175/434; 40%)	56% (245/434)	NM	65% (284/434)	39% (168/434)	NM	8% (30/434)	0% (0/434)	Chronic dacryocystitis risk factor for BK
South	Karnataka	Kotigadde S, 1992[19] (1985-1988)	0.9 M: 1F	age 0-9yrs M, 30-39yrs F	22% in FK (15/67)	NM	NM	NM	NM	NM	NM	Seasonal peak for FK: October
South	Chennai, Tamil Nadu	Venugopal PL, 1989[34] (1989)	M>F (% NM)	21-50	Mainly agricultural workers (% NM)	NM	NM	67%	NM	NM	NM	-
West	Jamnagar, Gujarat	Somabhai Katara R, 2013[35] (2006-8)	1.5M: 1F	20-70 (40%, 40/100) 31-60 in (77%, 20/26)	NM	NM	11% in FK (3/26)	62% in FK (16/26)	NM	NM	NM	Dacryocystitis/trachoma/conjunctivitis in BK (57%, 8/14)
West	Ahmedabad, Gujarat	Tewari A, 2012[36] (2007-8)	2M: 1F	21-60 (53%, 79/150)	Farmer/labourer/carpenter 42.7% (64/150)	Rural 30% (46/150)	90% (135/150)	18% (24/135)	NM	NM	0% (0/150)	-
West	Midnapore, West Bengal	Satpathi P, 2012[37] (?2012)	2M: 1F	60-69 (% NM)	55% (41/74)	NM	58% (43/74)	69.8% (30/43); 53% in FK (10/19)	NM	1.3% (1/74)	NM	-

Table 1 continued

India Zone	State & City	First author, publication year (study time period)	Male to Female ratio	Peak Age, years (%)	Agri-cultural work	Rural resid-ence	Trauma	Trauma with Vege-tative Matter	DM	Prior Topical Steroid Use	CL Wear	Other Risk Factors
West	Loni, Maharashtra	Deorukhkar S, 2012[38] (2004-9)	2M: 1F	21-30 Males; 31-40 Female	52.3% (281/537)	NM	60% (323/537)	NM	8% (44/537)	NM	NM	Cataract surgery 9% (51/537)
West	Saurashtra & Kutch, Gujarat	Kumar A, 2011[20] (2003-5)	1.6M: 1F	<50 yrs (64%, 129/200)	54% (108/200)	61% rural (123/200)	78% (157/200)	53% (83/157)	NM	25% (29/116)	NM	Seasonal peak Sept-Dec
West	Aurangabad, Maharashtra,	Pichare A, 2004[39] (?2004)	1.9M: 1F	21-30 (% NM)	NM	NM	NM	NM	NM	NM	NM	-
West	Sheikhpura, Patna	Kumari N, 2002[21] (?2000-2)	2M: 1F	21-50 (77% in FK)	82% (FK)	NM	82% (FK)	NM	7% (FK)	NM	NM	Seasonal peak Sept-Oct for FK
West	Mumbai, Maharashtra	Deshpande SD, 1999[40] (1988-1996)	M>F (% NM)	21-50 (79%)	88%	NM	90%	NM	NM	NM	NM	-
East	Bhubaneshwar, Odisha	Rautaraya B, 2014[41] (2006-10)	2.5M: 1F	60-69yrs in BK (23%, 69/303)	42% in BK (88/208)	NM	NM	42.3% in BK (78/180)	NM	NM	NM	Chronic dacryocystitis (in 22.3% of pneumococcal ulcers)
East	Sundarban, West Bengal	Bandyopadhyay S, 2012[40] (2007-2011)	1.6M: 1F (p<0.0001)	21-50 FK (67%, 269/399)	49% FK (196/399; p<0.0001)	86% FK rural (342/399; p<0.0001)	89% FK (354/399; p<0.0001)	74% (261/354; p<0.0001)	NM	16% (65/399)	NM	Seasonal peak in monsoon in Jun-Sept (48%, 192/399)

Table 1 continued

India Zone	State & City	First author, publication year (study time period)	Male to Female ratio	Peak Age, years (%)	Agri-cultural work	Rural resid-ence	Trauma	Trauma with Vege-tative Matter	DM	Prior Topical Steroid Use	CL Wear	Other Risk Factors
East	Dibrugarh, Assam	Nath R, 2011[22] (2007-9)	2M: 1F (FK)	41-50yrs (20%, 61/310)	100% in FK (157/157)	88% in FK (89/157) (138/157)	76% in FK (120/157)	86% in FK (103/120)	2% in FK (4/157)	0% (0/157)	0% (0/157)	Seasonal peak Jan-Feb (harvest season, 27% FK)
East	Kolkata, West Bengal	Saha S, 2009[42] (2008)	1.8 M: 1F (FK)	>50 yrs in FK (61%, 45/74)	NM	NM	47% in FK (35/74)	57% in FK (20/35)	NM	16% in FK (12/74)	NM	Prior ocular surgery in 12% (9/74 in FK)
East	Barrackpore, West Bengal	Basak S, 2005[23] (2001-3)	2.4M: 1F	31-40 (30%, 358/1198)	58% (691/1198)	78% rural (941/1198)	83% (994/1198; p<0.0001)	72% (715/994)	7.6% (92/1198)	19% (231/1198)	0.3% (4/1198)	Chronic dacryocystitis 2% (29/1198); Seasonal peak Nov-Dec (25%, 297/1198)

With regards to socio-demographic risk factors, most Indian studies have found that those affected by MK were predominantly male[4, 7-9, 15, 16, 18, 20, 21, 23, 25-28, 30, 35-39, 41-44], in the working age-group (i.e. 21-50 years of age)[7, 9, 15, 16, 18, 20-23, 25, 27, 28, 34, 38-40, 45], and engaged in agricultural work (studies summarized in Table 1). Ocular risk factors for MK include chronic dacryocystitis that is frequently linked to *Streptococcus pneumoniae* keratitis[46]. Exposure of the eye to mud or contaminated water was a greater risk factor for *Acanthamoeba* keratitis (AK) rather than contact lens wear.[31] Systemic risk factors for MK include diabetes mellitus.[8, 9, 15, 21-23, 38]

Early diagnosis and identification of the causative organism in MK allows the clinician to start the correct antimicrobial treatment, resulting in better visual outcome[47], and reduced risk of severe complications such as corneal perforation.[48] Although microbiological diagnosis using corneal scrape material is currently the reference standard for identifying the causative organism[49], slit lamp examination of the corneal ulcer may also yield clinical signs that may be indicative of either BK, FK or AK.[10]

1.3 Clinical Features of MK

Patients with acute MK usually present with an epithelial defect in the cornea with an underlying stromal infiltrate and signs of acute inflammation such as conjunctival injection, cells or flare in the anterior chamber and a hypopyon (see Figure 1a); these signs have been used to define acute BK or FK in clinical studies.[50, 51] "Classical" signs that have been reported to be typical of FK include feathery margins (Figure 1b), satellite lesions (Figure 1c), ring infiltrate (Figure 1d), and sometimes a dry surface with raised profile.[11, 52] An endothelial plaque and even hypopyon may be present in later stages of FK.[52] Few studies have specifically attempted to assess the statistical association of such specific clinical features with culture-positive microbial keratitis.[10, 12, 53-55] When looking at ulcer clinical features alone, corneal specialists (masked to the microbiological diagnosis) were able to correctly identify BK from FK 66% of the time in 70 MK photos (95% CI 63-68%, $p<0.001$).[10] Similarly in a previous study using live slit-lamp examination of patients, cornea specialists (again masked to microbiological diagnosis) were able to correctly determine the diagnosis of BK, FK or AK in 73% (n=54/74); correct prediction was much higher for BK (79%) than for FK (38%).[53]

Specific features that have been associated with FK are feathery margins ($p=0.002$), and in BK are epithelial plaques ($p=0.02$) or a wreath-like infiltrates in *Nocardia* keratitis ($p=0.005$).[10, 56] With

regards to BK, clinicians were able to successfully predict the gram stain result (i.e. positive or negative) in 46% of the 40 bacterial ulcers (95% CI 40-53%); for both BK and FK, the cornea specialists correctly identified the genus in 25% (95% CI 20-29%) and species in 20% (95% CI 15-25%).[10] Thomas *et al* assessed 360 bacterial and fungal corneal ulcers seen in South India and Ghana, to determine the diagnostic accuracy of clinical features.[55] Their final multivariable model found the following features were more associated with FK than BK: feathery/serrated margins (OR 3.4, 95% CI 2.1-5.6 p<0.01), raised profile (OR 2.3, 95% CI 1.4-3.7, p<0.01) and non-yellow colour (OR 2.8, 95% CI 1.3-6.0, p=0.01). Presence of fibrin in the anterior chamber was more associated with BK than FK (OR 0.4, 95% CI 0.2-0.8, p=0.01). In their univariate analysis, satellite lesions (OR 1.9, 95% CI 1.1-3.6, p=0.04) and a dry ulcer surface (OR 2.0, 95% CI 1.3-3.3, p<0.01) also occurred more frequently in FK, and hypopyon in BK (OR 0.5, 95% CI 0.3-0.8, p<0.01). However these features did not retain statistical significance in the final multivariable model. The most frequently cultured fungi in this study were *Fusarium* sp. followed by *Aspergillus* sp., and the main bacteria were *Streptococcus* sp., followed by coagulase-negative *Staphylococcus* sp.[14]

Oldenburg *et al* looked at clinical features that may help to differentiate hyaline versus dematiaceous fungi.[54] They found that raised lesions were more associated with dematiaceous fungi (OR 0.3, 95% CI 0.1-0.7, p=0.0065).[54] In addition, they found that compared to all other FK, *Aspergillus* ulcers were more likely to have a ring infiltrate (OR 14.0, 95% CI 1.1-178.7, p=0.042), especially those caused by *Aspergillus flavus* (OR 24.0, 95% CI 1.7-340.6, p=0.019), and that *Fusarium* ulcers were less likely to have an endothelial plaque (OR 0.2, 95% CI 0.03-0.97, p=0.046).[54]

For the diagnosis of AK, accuracy was significantly increased if a ring infiltrate was present (p<0.001)[12, 53], and this feature can occur in approximately 41%-45% of patients with microbiologically-proven AK (see Figure 1d).[31, 33] Also, peri-neuritis can occur in up to 57% of patients presenting with AK[47], but can also occur in BK caused by *Pseudomonas* sp.[57]

All of the above studies, show that although some features are more likely to occur in FK, or AK, their diagnostic accuracy remains limited and so other techniques, such as microbiological culture, remain important to correctly identify the causative organism.

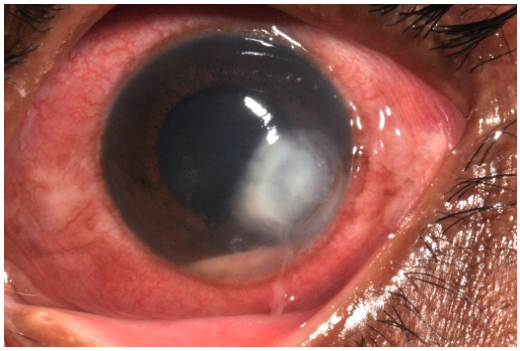


Fig. 1a) Ulcer with hypopyon in culture-positive *Streptococcus pneumoniae* keratitis

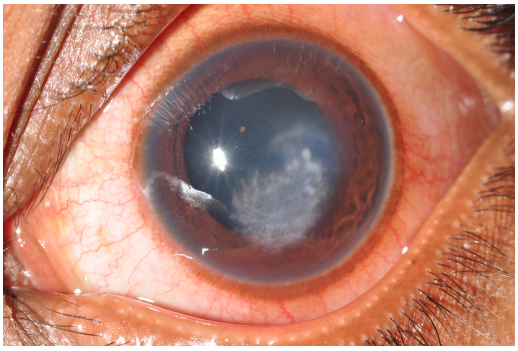


Fig. 1b) Feathery margins in culture-positive *Fusarium* keratitis

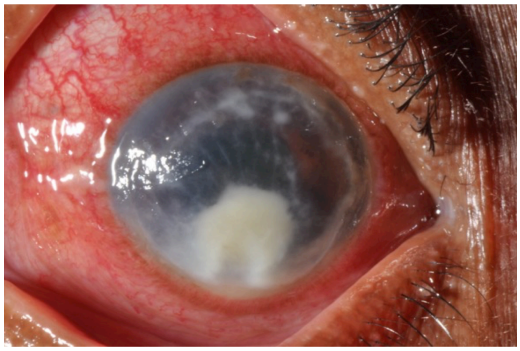


Fig. 1c) Satellite lesions seen superiorly in fungal keratitis

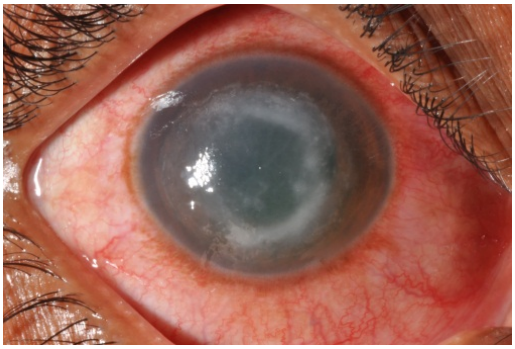


Fig. 1d) Ring infiltrate in culture-positive *Acanthamoeba* keratitis

Figure 1: Clinical features in bacterial, fungal and Acanthamoeba keratitis

1.4 Microbiological Diagnosis of MK

The current reference standard for the isolation of a causative organism in MK is direct staining of corneal scrape material and/or inoculation of media for microbiological culture.[49] However, even in high incidence settings for MK such as India the culture-positive rate of corneal scrapes can be as low as 37.5%.[8] Light microscopy positivity rates are often better than culture for the detection of fungi, however this requires experienced microbiology laboratory staff who are often not available in rural regions of India. Molecular methods such as PCR may also yield high positive rates for the detection of pathogens in corneal scrapes, but they can sometimes be too sensitive and detect environmental fungal spores that are not present in the corneal scrape sample resulting in false-positives therefore PCR has not as yet replaced culture/light microscopy as a diagnostic test in MK as yet.

In studies from India, the culture positive rate (i.e. number of corneal scrapes that were culture-positive for any organism out of all clinically suspected MK patients who underwent corneal scraping) varied from 37.5%[8] to 93.2%[22] (see Table 2). Of all patients presenting with clinically suspected MK, FK account for 8.3%[30] to 60.6%[22] of cases, BK for 8.6%[25] to 40.0%[29] of cases, and AK for 0.3%[23] to 2.0%[9] of cases. The most frequently detected fungus in FK in the North, East and West of India is *Aspergillus* sp. In contrast, in the warmer, more humid climate of South India, the most commonly cultured fungus from FK corneal specimens is *Fusarium* sp.

When performed by experienced personnel, light microscopy with the aid of stains such as gram stain for bacteria and 10% potassium hydroxide (KOH) or calcofluor white (CFW) for fungi can identify presence of an organism with high diagnostic accuracy. Compared to culture-positivity, the sensitivity of KOH or CFW for the detection of fungal filaments ranges from 62%[28] to 100%[37], and the specificity from 97%[28] to 100%[36] in Indian studies. Furthermore, although light microscopy is compared with culture in these studies, some authors have often noted that culture-negative samples are frequently light microscopy-positive for the organism, making this at times a better test for the diagnosis of FK or AK than culture, with an experienced microscopist.[7, 9, 27, 30]

However, in the remaining culture-negative, light microscopy-negative ulcers, the current reference standard microbiological techniques no longer provide a diagnosis, and clinical features are often not pathognomonic for BK, FK or AK. Therefore, alternative techniques are needed to aid in diagnosis of the pathogen. The development of high resolution imaging of the cornea, in the form of *in vivo* confocal microscopy (IVCM), has allowed direct visualization of organisms such as fungi and *Acanthamoeba* sp. within the patient's living cornea (see section 1.11).

Table 2: Summary of culture positive rates and major fungi/bacteria cultured in Indian microbial keratitis (MK) studies shown in Table 1. Note predominance of *Aspergillus* sp. as the primary fungal isolate in North India and *Fusarium* sp. in South India. (*total clinically suspected MK patients). Abbreviations: BK=bacterial keratitis, FK=fungal keratitis, AK=Acanthamoeba keratitis, NM=not mentioned.

First author, (Zone, City, State, Study period)	Total MK*	Total culture +ve	Total BK	Total FK	Total AK	Most frequently isolated fungus	Second fungal isolate	Third fungal isolate	Gram +ve bacterial isolates 1 & 2	Gram –ve bacterial isolates 1 & 2
Gupta A, 2014[25] (North: New Delhi, 2004-12)	209	38.3% (80)	8.6% (18)	29.7% (62)	0.5% (1)	Aspergillus sp.	Fusarium sp.	Candida sp.	NM	NM
Punia RS, 2014[26] (North: Chandigarh, Haryana & Punjab, 2003-12)	NM	NM	NM	NM	NM	Aspergillus sp.	Fusarium sp.	Curvularia sp.	NM	NM
Sharma V, 2008[7] (North: Ujjain, Madhya Pradesh, 2006-7)	201	NM	NM	20.9%* (42)	NM	Aspergillus sp.	Candida sp.	Curvularia sp.	NM	NM
Chander J, 2008[27] (North: Chandigarh, Haryana & Punjab, 1999-2003)	154	NM	NM	41.5% (64)	NM	Aspergillus sp.	Fusarium sp.	Candida sp.	NM	NM
Chowdhary A, 2005[28] (North: New Delhi, 1999-2001)	485	NM	NM	39.4%* (191)	NM	Aspergillus sp.	Curvularia sp.	Fusarium sp.	NM	NM
Bashir G, 2005[29] (North: Srinagar, Jammu & Kashmir, 1999-2001	80	52.5% (42)	40.0% (32)	12.5% (10)	NM	Aspergillus fumigatus	Fusarium sp.	NM	Streptococcus pneumoniae	NM
Chander J, 1994[30] (North: Chandigarh, Haryana & Punjab, 1988-1994)	730	NM	NM	8.3% (61)	NM	Aspergillus sp.	Fusarium sp.	Curvularia sp. & Candida sp.	NM	NM

Table 2 continued

First author, (Zone, City, State, Study period)	Total MK*	Total culture +ve	Total BK	Total FK	Total AK	Most frequently isolated fungus	Second fungal isolate	Third fungal isolate	Gram +ve bacterial isolates 1 & 2	Gram –ve bacterial isolates 1 & 2
Ranjini CY, 2016[8] (South: Bangalore, Karnataka, 2012-14)	312	37.5% (117)	18.9% (59)	18.6% (58)*	NM	Fusarium sp.	Aspergillus *sp.*	Curvularia *sp.*	Staph. aureus Strep. pneumoniae	Pseudomonas aeruginosa Klebsiella pneumoniae
Manikandan P, 2013[17] (South: Coimbatore, Tamil Nadu, 2005-8)	3176	54.4% (1727)	17.6% (560)	34.2% (1087)	AK 0.5% (15)	Aspergillus sp.	NM	NM	NM	NM
Gopinathan U, 2009[9] (South: Hyderabad, Telangana, 1991-2001)	5897	60.4% (3563)	31.3% (1849)	23.1% (1360)	AK 2.0% (118)	Fusarium sp.	Aspergillus sp.	Curvularia sp.	Staph epidermidis Strep pneumoniae	Pseudomonas sp. Moraxella sp.
Jayahar Bharathi M, 2007[15] (South: Tirunelveli, Tamil Nadu, 1999-2002)	3183	70.6% (2247)	32.8% (1043)	34.4% (1095)	1.0% (33)	Fusarium sp.	Aspergillus sp.		Strep pneumoniae Staph epidermidis	Pseudomonas sp. Enterobacter sp.
Manikandan P, 2004[32] (South: Coimbatore, Tamil Nadu, 1997-2003)	4519	66.6% (3008)	NM	NM	0.7%* (32)	NM	NM	NM	NM	
Gopinathan U, 2002[18] (South: Telangana, 1991-2000)	NM	(3399)	NM	FK 39.8% (1352/3399)	NM	Fusarium sp.	Aspergillus sp.	Curvularia sp.	NM	NM
Srinivasan, M, 1997[13] (South: Madurai, Tamil Nadu, 1994)	434	68.4% (297)	32.2% (140)	32.0% (139)	0.7% (3)	Fusarium sp.	Aspergillus sp.	Lasiodiplodia theobromae	Strep pneumoniae Corynebacterium xerosis	Pseudomonas sp. Moraxella sp./Haemophilus influenza

Table 2 continued

First author, (Zone, City, State, Study period)	Total MK*	Total culture +ve	Total BK	Total FK	Total AK	Most frequently isolated fungus	Second fungal isolate	Third fungal isolate	Gram +ve bacterial isolates 1 & 2	Gram –ve bacterial isolates 1 & 2
Kotigadde S, 1992[19] (South: Karnataka, 1985-1988)	295	38.3% (113)	15.6% (46)	22.7% (67)	NM	Aspergillus sp.	Candida sp.	Rhizopus nigricans & Pseudallescheria boydii	Staph aureus Coagulase-negative staph.	Pseudomonas aeruginosa Klebsiella pneumoniae
Venugopal PL, 1989[34] (South: Chennai, Tamil Nadu, 1989)	698 FK	NM	NM	46.1% (322)	NM	Aspergillus sp.	Acremonium sp.	Curvularia sp.	NM	NM
Somabhai Katara R, 2013[35] (West: Jamnagar, Gujarat, 2006-8)	100	40.0% (40)	14.0% (14)	26.0% (26)	NM	Aspergillus sp.	Fusarium sp.	Candida sp.	Streptococcus pneumoniae Other Strep. sp.	Pseudomonas aeruginosa Proteus sp.
Tewari A, 2012[36] (West: Ahmedabad, Gujarat, 2007-8)	150	59.3% (89)	38.7% (58)	20.7% (31)	NM	Aspergillus sp.	Fusarium sp.	Curvularia sp.	Staph aureus Coagulase-negative staph.	Pseudomonas sp. Klebsiella sp.
Satpathi P, 2012[37] (West: Midnapore, West Bengal, ?2012)	74	52.7% (39)	27.0% (20)	25.7% (19)	NM	Aspergillus sp.	Fusarium sp.	Phoma sp.	Staph aureus Strep pneumoniae	Pseudomonas aeruginosa Acinetobacter baumanii
Deorukhkar S, 2012[38] (West: Maharashtra,)	852	63.0% (537)	26.5% (226)	36.5%* (311)	NM	Aspergillus sp.	Candida sp.	Penicillium sp.	Strep pneumoniae Staph sp.	Pseudomonas sp. Klebsiella sp.
Kumar A, 2011[20] (West: Saurashtra/Kutch, Gujarat, 2003-5)	200	55.0% (110)	26.5% (53)	22.5% (45)	0.5% (1)	Fusarium sp.	Aspergillus sp.	Curvularia sp.	Staph aureus Strep pneumoniae	Pseudomonas aeruginosa E. Coli
Pichare A, 2004[39] (West: Aurangabad, Maharashtra, ?2004)	60	38.3% (23)	16.7% (10)	20.0% (12)	NM	Fusarium sp.	NM	NM	Staph aureus	NM

Table 2 continued

First author, (Zone, City, State, Study period)	Total MK*	Total culture +ve	Total BK	Total FK	Total AK	Most frequently isolated fungus	Second fungal isolate	Third fungal isolate	Gram +ve bacterial isolates 1 & 2	Gram –ve bacterial isolates 1 & 2
Kumari N, 2002[21] (West: Sheikhpura, Patna, ?2000-2)	204 FK	NM	NM	37.2% (76)	NM	Aspergillus sp.	Candida sp.	Fusarium sp. Penicillium sp.	NM	NM
Deshpande SD, 1999[40] (West: Mumbai, Maharashtra, 1988-1996)	1010 FK	NM	NM	36.3% (367)	NM	Aspergillus sp.	Candida sp.	Fusarium sp.	NM	NM
Rautaraya B, 2014[41] (East: Bhubaneshwar, Odisha, 2006-10)	1417	72.2% (1023)	21.4% (303)	28.6% (405)	1.5% (21)	NM	NM	NM	Streptococcus pneumoniae Staph. Sp.	Pseudomonas sp. Enterobacteriaceae
Rautaraya B, 2011[43] (East: Bhubaneshwar, Odisha, 2006-9)	997	74.6% (744)	23.4% (233)	26.5% (264)	1.0% (10)	Aspergillus sp.	Fusarium sp.	Acremonium sp.	Staph. Sp. Corynebacterium sp.	Pseudomonas sp. Acinetobacter sp.
Bandyopadhyay S, 2012[40] (East: Sundarban, West Bengal, 2007-2011)	928	72.9% (677)	29.3% (272)	39.1% (363)	0.6% (6)	Aspergillus sp.	Fusarium sp.	Curvularia sp.	NM	NM
Nath R, 2011[22] (East: Dibrugarh, Assam, 2007-9)	310	93.2% (289)	31.6% (98)	60.6%* (188)	1.0% (3)	Fusarium sp.	Aspergillus sp.	Curvularia lunata	NM	NM
Saha S, 2009[42] (East: Kolkata, West Bengal, 2008)	289	NM	NM	FK 38.1% (110/289)	NM	Aspergillus sp.	Candida albicans	Fusarium sp.	NM	NM
Basak S, 2005[23] (East: Barrackpore, West Bengal, 2001-3)	1198	67.7% (811)	15.3% (184)	42.5% (509)	0.3% (4)	Aspergillus sp.	Fusarium sp.	Penicillium sp.	NM	NM

1.5 Comparison with MK in the UK and other countries of similar socio-economic status or climate to India

In comparison to India, studies of the epidemiology of MK in the UK show the main organisms cultured from corneal scrapes in MK are coagulase-negative staphylocci and *Staphylococcus aureus* as the main gram-positive bacteria, with *Pseudomonas aeruginosa* and either *Moraxella* sp. or *Serratia* sp. as the main gram-negative bacteria (see Table 3). The most frequently cultured fungus was *Candida* sp. (especially *Candida albicans*), and followed by *Fusarium* sp. or *Aspergillus* sp. The most common risk factor associated with MK in UK studies was contact-lens wear (see Table 4), which is in contrast to Indian studies where trauma to the cornea, often with vegetative matter, is more frequent.

Several South American countries share a similar tropical and humid climate to South India, and similar socio-economic status (i.e. Brazil, Argentina, Paraguay and India are within the same World Bank low and middle-income country grouping). South American studies have shown the main fungal pathogen isolated in MK to be *Fusarium* sp. (similar to south Indian studies), followed by *Aspergillus* sp. (see Table 5) Bacterial pathogens were more likely to be *Staphylococci* (coagulase-negative *Staphylococci*, *Staphylococcus aureus* or *Staphylococcus epidermidis*), followed by *Streptococcus pneumoniae*. *Pseudomonas aeruginosa* was the most commonly cultured gram-negative bacterium in these South American studies, as also found in UK and Indian studies. Risk factors for developing MK included trauma, especially with vegetative matter in the harvest season, in studies from Argentina and Brazil, as well as an increased contact lens use compared to Indian studies (see Table 6).

Studies from African countries on a similar latitude to Tamil Nadu in South India (i.e. Ghana, Sierra Leone) also show filamentous fungi such as *Aspergillus* sp. and *Fusarium* sp. as the predominant fungus in MK isolates. In terms of the main bacterial pathogens in African studies, *Staphylococci* and *Streptococcus pneumoniae* were the most frequently isolated gram-positives, and *Pseudomonas aeruginosa* the main gram-negative organism (see Table 7). Although contact lens wear was reported in some studies as an important risk factor,[58, 59] corneal trauma associated with agricultural work was reported in Egypt and Nigeria,[58, 60] and use of contaminated topical medication or ocular cosmetics was associated with MK in Ethiopia (Table 8).[61, 62]

1.6 Microbiology of Moderate/Severe MK

Since moderate to severe corneal ulcers (i.e. stromal infiltrate diameter $\geq$ 3mm) frequently have worse outcomes (e.g. corneal perforation)[63], we chose to investigate these ulcers in this PhD. Few studies have investigated the microbiological profile of severe keratitis and found that environmental organisms are more likely to cause these ulcers. In the case of bacterial keratitis, severe keratitis with vision loss was more likely to be caused by *Pseudomonas* sp. rather than *Staphylococcal* species in a pan-Australian study.[64] This study, which included cases from the tropical and temperate regions of Austrialia, found that all *Staphylococci* were more likely to be culture from corneal ulcers in temperate parts of Australia and environmental pathogens, e.g. *Pseudomonas* sp., were more likely in the tropical regions. With regards to fungal keratitis, in South India, more severe keratitis was more likely to be caused by *Fusarium* sp. or *Aspergillus* sp.[65]

As such, the microbiological spectrum of organisms that are cultured from moderate to severe keratitis in South India will be different to the spectrum of organisms cultured from all ulcer sizes presenting to the Cornea Clinic.

Table 3: Summary of culture positive rates and major fungi/bacteria cultured in UK and Republic of Ireland microbial keratitis studies.

Search methodology: PubMed database (https://www.ncbi.nlm.nih.gov/pubmed/) accessed on 8 July 2018 using search terms "epidemiology", or "incidence" or "prevalence", combined with "corneal ulcer" or "bacterial keratitis" or "fungal keratitis" or "mycotic keratitis", and "United Kingdom", "England", "Scotland", "Wales" or "Ireland"; review articles excluded). Abbreviations: MK=microbial keratitis, BK=bacterial keratitis, FK=fungal keratitis, AK=acanthamoeba keratitis, NM=not mentioned, M=male, F=female, CL=contact lens wear, DM=diabetes mellitus. *Total MK=Total clinically suspected MK patients.

First author, (City, Country, Study period)	Total MK*	Total culture +ve	Total BK	Total FK	Total AK	Most frequently isolated fungus	Second fungal isolate	Third fungal isolate	Gram +ve bacterial isolates 1 & 2	Gram –ve bacterial isolates 1 & 2
Awan, MA, 2010, Glasgow, UK (1995-8 & 2004-7)[66]	1155	30.5% (352/1155)	29.9% (345/1155)	NM	NM	NM	NM	NM	Staph aureus Coag-neg staph sp.	Pseudomonas aeruginosa Non-lactose fermenting coliforms
Melia, B, 2008, Hull, UK (2008)[67]	100	22%	NM	NM	NM	Fusarium moniliforme	NM	NM	Staphylococcus aureus	Pseudomonas sp. Klebsiella sp.
Kaye R, 2013, Liverpool, UK (1995-2010)[68]	2418	NM	35.7%	NM	NM	NM	NM	NM	Coag-neg staph sp. Streptococci sp.	Enterobacteriaceae Pseudomonas sp.
Tan, SZ, 2017, Manchester, UK (2004-15)[69]	4229	32.6% (1379/4229 patients; 1539 organisms grown)	1394/1539 (including mixed infections)	109/1539 (including mixed infections)	36/1539 (including mixed infections)	Candida sp.	Fusarium sp.	NM	Coag-neg staph sp. Staph aureus	Pseudomonas sp. Moraxella sp.
Otri, AM, 2013, Nottingham, UK (2007-10)[70]	192	41.7% (53/127)	34.6% (44)	0%	7% (9)	Nil	Nil	Nil	Staph aureus Strep pneumoniae	Pseudomonas aeruginosa Moraxella sp.
Orlans, HO, 2011, Oxford, UK (1999-2009)[71]	467	54.0% (252)	54.0% (252)	0%	0%	Nil	Nil	Nil	Coag-neg staph sp. Staph aureus	Pseudomonas sp. Moraxella sp.

Table 3 continued

First author, (City, Country, Study period)	Total MK*	Total culture +ve	Total BK	Total FK	Total AK	Most frequently isolated fungus	Second fungal isolate	Third fungal isolate	Gram +ve bacterial isolates 1 & 2	Gram –ve bacterial isolates 1 & 2
Stapleton, F, 2017, London, UK & Sydney, Australia (London: 2003-5; Sydney: 2003-4)[72]	166	47.5% (116/255)	41.9% (107)	1.6% (4)	2.0% (5)	Fusarium sp. (Fusarium dimerum)	Acremonium sp.	Candida sp.	Coag-neg staph sp. Staph aureus	Pseudomonas sp. Serratia marcescens
Ong, HS, 2016, London, UK (2007-14)[73]	112 (FK only)	NM	NM	N=112 (100%)	NM	Fusarium sp.	Candida sp.	Aspergillus sp.	NM	NM
Tuft, SJ, 2009, UK (2003-2005)[74]	NM	NM	NM	FK only study: 90% (35/39)	NM	Candida sp (Candida albicans)	Aspergillus sp. (Aspergillus fumigatus)	Scedosporium apiospermum	NM	NM
Tuft, SJ, 2000, London, UK (1984-1999)[75]	1239	NM	100% (Only Bacterial Culture +ves included in study)	NM	NM	NM	NM	NM	Staphylococcus sp. Streptococcus sp.	Pseudomonas sp. Moraxella sp.
Shalchi, Z, 2011, East Kent, UK (1999-2008)	440	34.2% (163/440)	36.8% (162/440)	1.1% (5/440)	1.1% (5/440)	NM	NM	NM	Staph aureus Coag-neg staph sp.	Pseudomonas aeruginosa Serratia sp.
Farrell, S, 2017, Dublin, Ireland (?2011-2017)[76]	42 (FK only)	NM	NM	N=42 (100%)	NM	Aspergillus sp.	Candida sp.	Fusarium sp.	NM	NM

Table 4: Summary of the main epidemiological risk factors associated with clinically suspected microbial keratitis (MK) in the UK & Republic of Ireland. NB: Table 3 contains data on the proportion of BK, FK and AK in each study, and specific organisms cultured.

City, Country	First author, publication year (study time period)	Male to Female ratio	Peak Age, years (%)	Agri-cultural work	Rural resid-ence	Trauma	Trauma with Vege-tative Matter	DM	Prior Topical Steroid Use	CL Wear	Other Risk Factors
Glasgow, UK	Awan, MA, 2010 (1995-8 & 2004-7)[66]	NM	NM	NM	NM	NM	NM	NM	NM	NM	
Hull, UK	Melia, B, 2008 (2008)[67]	1M : 2F	Mean age 37 years	NM	NM	NM	NM	NM	NM	100%	
Liverpool, UK	Kaye, R, 2013 (1995-2010)[68]	NM	NM	NM	NM	NM	NM	NM	NM	NM	Recurrent bacterial keratitis in the same patient associated with Staph aureus
Manchester, UK	Tan, SZ, 2017 (2004-15)[69]	NM	n age 45.9years (SD 21.0)	NM	NM	NM	NM	NM	NM	NM	

Table 4 continued

City, Country	First author, publication year (study time period)	Male to Female ratio	Peak Age, years (%)	Agri-cultural work	Rural resid-ence	Trauma	Trauma with Vege-tative Matter	DM	Prior Topical Steroid Use	CL Wear	Other Risk Factors
Nottingham, UK	Otri, AM, 2013 (2007-10)[70]	1M : 1.1F	Average age 52.8 years (SD = 22.1)	NM	NM	2.8%	NM	0.7%	40%	26.5%	Peak incidence in Summer (June-Aug). Prior ocular surgery 20.2%
Oxford, UK	Orlans, HO, 2011 (1999-2009)[71]	NM	NM	NM	NM	NM	NM	NM	NM	NM	
London, UK (& Sydney, Australia)	Stapleton, F, 2017 (London: 2003-5; Sydney: 2003-4)[72]	1M : 1.8F	25-50 years (66%)	0%	NM	NM	NM	NM	NM	100% (study based on CL users)	Lack of handwashing before CL use (OR 1.8, 95% CI 1.6-2.0, p<0.001) Nelfilcon A CL material (OR 3.98, 95% CI 3.6-4.4, p<0.001) Overnight CL wear (OR 1.83, 95% CI 1.6-2.1, p<0.001) Smoking (OR 1.29, 95% CI 1.1-1.6, p=0.016)
London, UK	Ong, HS, 2016 (2007-14)[73] FK only study	1M : 1.4F (FK)	Median age 47.2 years (FK)	NM	NM	11.6% (FK)	NM	4.5%	32.1% (FK)	57.1% (FK)	Ocular surface disease (22.3%); prior ocular surgery (22.3%)

Table 4 continued

City, Country	First author, publication year (study time period)	Male to Female ratio	Peak Age, years (%)	Agri-cultural work	Rural resid-ence	Trauma	Trauma with Vege-tative Matter	DM	Prior Topical Steroid Use	CL Wear	Other Risk Factors
UK	Tuft, SJ, 2009 (2003-2005)[74]	1M : 1.6F	Median 57 yrs (range 16–95)	NM	NM	69%	NM	NM	52.6%	7.7%	Yeast infections more common in females, filamentary fungi in males. Hypopyon more likely in filamentary fungal keratitis. Prior ocular surface disease 64%
London, UK	Tuft, SJ, 2000 (1984-1999)[75]	NM	NM	NM	NM	NM	NM	NM	NM	NM	
East Kent, UK	Shalchi, Z, 2011 (1999-2008)[77]	1M : 1.3F	Bimodal peaks: 31–40-years & 81–90-years	NM	NM	NM	NM	NM	NM	NM	
Dublin, Ireland	Farrell, S, 2017 (?2011-2017)[76] FK only study	1.3M : 1F (FK)	Mean age 7.4 years (SD 20.7) (FK)	NM	NM	19.0% (FK)	9.5% (4/42) (FK)	NM	28.6% (FK)	FK: 40.5% (including bandage CL wear)	Prior ocular surgery (e.g. PK in 8, recent Collagen cross-linking)

Table 5: Summary of culture positive rates and major fungi/bacteria cultured in South American studies of microbial keratitis studies.

Search methodology: PubMed database (https://www.ncbi.nlm.nih.gov/pubmed/) accessed on 8 July 2018 using search terms "epidemiology", or "incidence" or "prevalence", combined with "corneal ulcer" or "bacterial keratitis" or "fungal keratitis" or "mycotic keratitis", and "South America"; review articles excluded). Abbreviations: MK=microbial keratitis, BK=bacterial keratitis, FK=fungal keratitis, AK=acanthamoeba keratitis, NM=not mentioned, M=male, F=female, CL=contact lens wear, DM=diabetes mellitus. *Total MK=Total clinically suspected MK patients.

First author, (City, Country, Study period)	Total MK*	Total culture +ve	Total BK	Total FK	Total AK	Most frequently isolated fungus	Second fungal isolate	Third fungal isolate	Gram +ve bacterial isolates 1 & 2	Gram –ve bacterial isolates 1 & 2
Refojo, N, 2016[78]; Buenos Aires, Argentina (2007-2013)	157	96.0% (151 FK only)	NM	100% (FK only study)	NM	Fusarium sp.	Aspergillus sp.	Curvularia sp.	NM	NM
Cariello AJ, 2011[79]; São Paulo, Brazil (1975-2007)	6,804 total culture +ve MK	48.6% (3,309)	39.7% (2,699)	5.3% (364)	3.6% (246)	NM	NM	NM	NM	NM
Ibrahim MM, 2011[80]; Ribeirão Preto & São Paulo, Brazil (2003-6)	118	62.2% (73)	55.4% (66)	43.7% (52)	0.8% (1)	Fusarium sp.	Aspergillus sp.	Penicillium sp.	Staph epidermidis/ Staph aureus Strep pneumoniae	Pseudomonas aeruginosa Serratia sp.
Ibrahim MM, 2009[81] São Paulo, Brazil (2000-04)	66 (FK alone)	NM	NM	NM	NM	Fusarium sp.	Aspergillus sp.	Candida sp.	NM	NM
Nentwich MM, 2015[82]; Asunción, Paraguay (2009-11)	48	NM	NM	64.5% (31)	NM	Fusarium sp.	NM	NM	NM	NM
Laspina, F, 2004[83]; Asunción, Paraguay (1988-2001)	660	79.0% (524)	40% (267)	21% (136)	NM	Acremonium sp.	Fusarium sp.	NM	Coag-neg staph sp. Staph aureus	Pseudomonas aeruginosa
Furlanetto RL, 2010[84]; Uberlandia, Brazil (2001-4)	65	49.23% (32/65)		27.7% (18/32)	NM	Fusarium sp.	NM	NM	Strep pneumoniae	NM

Table 6: Summary of the main epidemiological risk factors associated with clinically suspected microbial keratitis (MK) in South American studies. NB: Table 5 contains data on the proportion of BK, FK and AK in each study, and specific organisms cultured.

City, Country	First author, publication year (study time period)	Male to Female ratio	Peak Age, years (%)	Agri-cultural work	Rural resid-ence	Trauma	Trauma with Vege-tative Matter	DM	Prior Topical Steroid Use	CL Wear	Other Risk Factors
Buenos Aires, Argentina	Refojo, N, 2016[78] (2007-2013)	2.8M : 1F	31-40 (Males); 61-70 (Females)	NM	NM	40%	NM	4%	NM	9%	Herpetic abscess 5%
São Paulo, Brazil	Cariello AJ, 2011[79] (1975-2007)	1.5M : 1F	42.1 ± 21.4 years	NM	NM	16.4% (1,118)	3.8x increased risk FK	NM	NM	CL wear 1.7x increased risk AK (p<0.01)	Prior ocular surgery 22.4% (1,524)
Ribeirão Preto & São Paulo, Brazil	Ibrahim MM, 2011[80] (2003-6)	2.7M : 1F For FK 6.4M : 1F	Mean age BK 50.6 ± 21.0 years Mean age FK 41.18 ± 17.49 years	FK 20% (24). BK 8% (9)	FK 19% (22) BK 8.5% (10)	36% (36/107)	FK 79% (22/28) BK 20% (2/10)	6.8% (8/118)i.e. 5.9% (7/118) in BK, 0.8% (1/118) in FK	NM	NM	Higher BK & FK in May and Nov (Harvest season)
São Paulo, Brazil	Ibrahim MM, 2009[81] (2000-04)	4M : 1F	Mean age 41yrs +/- 16 years	NM	NM	40%	NM	NM	NM	NM	Prior quinolone use 73%; prior antifungal use 30%

Table 6 continued

City, Country	First author, publication year (study time period)	Male to Female ratio	Peak Age, years (%)	Agri-cultural work	Rural resid-ence	Trauma	Trauma with Vege-tative Matter	DM	Prior Topical Steroid Use	CL Wear	Other Risk Factors
Asunción, Paraguay	Nentwich MM, 2015[82] (2009-11)	1.7M : 1F	NM	NM	NM	43.8%	NM	NM	NM	NM	Median time to presentation 7 days (range 1-30 days)
Asunción, Paraguay	Laspina, F, 2004[83] (1988-2001)	1.9M : 1F	30-59 (for FK)	Risk for FK	NM	50% (esp for FK)	25% trauma with foreign body	NM	NM	NM	>1 week delay in diagnosis in most patients Self-medication as a risk for FK
Uberlandia, Brazil	Furlanetto RL, 2010[84] (2001-4)	2.6M : 1F	Mean 45.9 years (range 2-83 years)	NM	NM	40% (28/65)	NM	NM	NM	NM	

Table 7: Summary of culture positive rates and major fungi/bacteria cultured in African studies of microbial keratitis.

Search methodology: PubMed database (https://www.ncbi.nlm.nih.gov/pubmed/) accessed on 8 July 2018 using search terms "epidemiology", or "incidence" or "prevalence", combined with "corneal ulcer" or "bacterial keratitis" or "fungal keratitis" or "mycotic keratitis", and "Africa"; review articles excluded). Abbreviations: MK=microbial keratitis, BK=bacterial keratitis, FK=fungal keratitis, AK=acanthamoeba keratitis, NM=not mentioned, M=male, F=female, CL=contact lens wear, DM=diabetes mellitus. *Total MK=Total clinically suspected MK patients.

First author, (City, Country, Study period)	Total MK*	Total culture +ve	Total BK alone	Total FK alone	Total AK	Most frequently isolated fungus	Second fungal isolate	Third fungal isolate	Gram +ve bacterial isolates 1 & 2	Gram –ve bacterial isolates 1 & 2
Zbiba W, 2016 (Cap Bon, Tunisia, 2011-16)[85]	230	87.4% (201)	40% (92)	32% (74)	6% (14)	NM	NM	NM	NM	NM
Cheikhrouhou, F, 2014 (Sfax, Tunisia, 1995-2012)[59]	483	NM	NM	12.4% (60/483)	NM	Fusarium sp. (Fusarium solani)	Aspergillus sp. (Aspergillus fumigatus)	Candida sp. (Candida albicans)	NM	NM
Badawi, AE, 2017 (Mansoura, Egypt, 2013-15)[58]	245	44.5% (110)	40%	45.5%	4.5%	*Aspergillus* sp.	*Fusarium* sp	Candida sp.	Staph. aureus	Pseudomonas aeruginosa
Teweldemedhin, M, 2017 (Quiha, Tigray, Ethiopia, Sept-Dec 2015)[62]	NM	NM	80.7% (46)	NM	NM	NM	NM	NM	Coag-neg staph sp. Staph aureus	Pseudomonas aeruginosa E. Coli
Gebremariam TT, 2015 (Jimma, Ethiopia, Jan-Jun 2012)[61]	24	83% (20/24)	100% (20)	0%	0%	Nil	Nil	Nil	Staph aureus Strep pneumoniae	Pseudomonas aeruginosa Serratia marcescens
El Shabrawy, RM, 2013 (Zagazig, Egypt, Jan 2012-Jun 2012)[86]	350 (but only 60 included)	NM	NM	55% (33/60)	NM	Penicillium sp.	Aspergillus fumigatus	Candida sp.	NM	NM

Table 7 continued

First author, (City, Country, Study period)	Total MK*	Total culture +ve	Total BK alone	Total FK alone	Total AK	Most frequently isolated fungus	Second fungal isolate	Third fungal isolate	Gram +ve bacterial isolates 1 & 2	Gram –ve bacterial isolates 1 & 2
Oladigbolu, K, 2013 (Shika-Zaria Nigeria, 1995-2005)[60]	228	58% (98/169)	NM	15.8%	NM	NM	NM	NM	Staph aureus Strep pneumoniae	Pseudomonas aeruginosa
Ubani, UA, 2009 (Aba, Nigeria, 2005-6)[87]	55	NM	100% (55) BK only study	NM	NM	NM	NM	NM	Staph aureus Staph albus	Pseudomonas aeruginosa E. Coli
Capriotti, JA, 2009 (Makeni, Sierra Leone, Jan 2005 & Jan 2006)[88]	73	94.5% (69/73)	82.2% (60/73)	35.6% (26/73)	0%	Aspergillus niger	Rhizopus sp.	Penicillium sp. Fusarium sp.	Staph aureus Coag-neg staph sp.	Pseudomonas aeruginosa E.Coli
Leck, AK, 2002 (Accra, Ghana, 1999-2001)[14]	290	50.3% (144/290)	12.4% (36)	36.2% (105)	0.3% (1)	Fusarium sp.	Aspergillus sp. (Aspergillus flavus)	Curvularia sp.	Strep pneumoniae Coag-neg staph sp.	Pseudomonas aeruginosa Acinetobacter sp.
Wani, MG, 2001 (Harare, Zimbabwe, Oct 1994-Mar 1995)[89]	38	36.7% (11/30)	36.7% (11/30)	0%	0%	Nil	Nil	Nil	Staph epidermidis Streptococcus sp.	E.Coli Pseudomonas aeruginosa
Burton, MJ, 2011 (Moshi, Tanzania, 2008-2010)[90]	170	54% (31/57)	26.3% (15/57)	28.1% (16/57)	0%	Filamentary fungi (species unidentified)	Candida sp.	NM	Staph epidermidis Strep viridans	Pseudomonas aeruginosa

Table 8: Summary of the main epidemiological risk factors associated with clinically suspected microbial keratitis (MK) in African studies. NB: Table 7 contains data on the proportion of BK, FK and AK in each study, and specific organisms cultured.

City, Country	First author, publication year (study time period)	Male to Female ratio	Peak Age, years (%)	Agri-cultural work	Rural resid-ence	Trauma	Trauma with Vege-tative Matter	DM	Prior Topical Steroid Use	CL Wear	Other Risk Factors
Cap Bon, Tunisia	Zbiba, W, 2016 (2011-16)[85]	1.5M : 1F	FK: <50 years (71.6%) BK >50 years (65.2%) AK <50 years (92.8%)	NM	FK 73% AK 79% BK 35%	51.3% (81% in FK)	NM	16% (FK 16%, BK 15%)	10.9% (FK 13.5%, BK 12%, AK 0%)	9.5% (FK 0%, BK 13%, AK 71.4%)	Late diagnosis >30 days (13%)
Sfax, Tunisia	Cheikhrouhou, F, 2014 (1995-2012)[59]	1.6M : 1F (for FK)	Mean age 47 years (FK only)	NM	FK: 57.5%	FK: 61.6%	FK: 42.4%	FK: 5%	FK: 18.3%	FK: 3.3% (2)	FK: Prior ocular surgery (11.7%; 7)
Mansoura, Egypt	Badawi, AE, 2017 (2013-15)[58]	1.9M : 1F	40-59 years	44.1%	NM	51.4%	NM	15.1%	5.3%	2.4%	
Quiha, Tigray, Ethiopia	Teweldemedhin, M, 2017 (Sept-Dec 2015)[62]: data for all ocular infections incl keratitis	1M : 1.3F	Mean age 37.8 years (SD 22.9 yrs)	NM	58.5% (155)	83.3% (70)	NM	NM	NM	15.8% (9/57)	No formal education 45.2% (122); application of ocular cosmetics OR of BK of 4.7 (1.6–13.9), p<0.01
Jimma, Ethiopia	Gebremariam TT, 2015 (Jan-Jun 2012)[61]	7M : 1F	NM	NM	NM	37.5% (9)	NM	NM	NM	NM	Contaminated medicine use (17%; 4/24)
Zagazig, Egypt	El Shabrawy, RM, 2013 (Jan 2012-Jun 2012)[86]	NM	NM	NM	NM	FK: 63.6% (21/33)	NM	FK: 18.2% (6/33)	FK: 6.1% (2/33)	FK: 12.1% (4/33)	Chronic liver disease (30.3%; 10/33)

Table 8 continued

City, Country	First author, publication year (study time period)	Male to Female ratio	Peak Age, years (%)	Agri-cultural work	Rural resid-ence	Trauma	Trauma with Vege-tative Matter	DM	Prior Topical Steroid Use	CL Wear	Other Risk Factors
Shika-Zaria Nigeria	Oladigbolu, K, 2013 (1995-2005)[60]	1.3M : 1F	Peak <40 years (range 15 months-66 years)	14.5% (33)	NM	51.3% (117)	15.8% (36)	NM	5.7% (13)	0.4% (1)	
Aba, Nigeria	Ubani, UA, 2009 (2005-6)[87]	1.2 M : 1F (BK only)	18-40 years (36.4%) BK only	NM	NM	NM	NM	NM	NM	NM	
Makeni, Sierra Leone	Capriotti, JA, 2008 (Jan 2005 & Jan 2006)[88]	NM	Range: 7 months–91 years	NM	NM	NM	NM	NM	NM	NM	
Accra, Ghana	Leck, AK, 2002 (1999-2001)[14]	NM	NM	NM	NM	NM	NM	NM	NM	NM	
Harare, Zimbabwe	Wani, MG, 2001 (Oct 1994-Mar 1995)[89]	1.4M : 1F	Median age 33.7 years (range 10-95yrs)	NM	NM	36.4% (8/22)	NM	NM	NM	NM	
Moshi, Tanzania	Burton, MJ, 2011 (2008-2010)[90]	1.2M : 1F	Median age 46 years (Interquartile Range 25–59 years)	NM	NM	24% (41)	NM	NM	17.1% (29)	NM	

1.7 Microbiology of main MK pathogens in South India

The main pathogens cultured from moderate-to-severe corneal ulcers in South India are the filamentous fungi *Fusarium* sp. and *Aspergillus sp.*, the *Acanthamoeba* parasite, and the bacterial pathogen *Streptococcus pneumoniae*. Here we will discuss the microbiology of each of these pathogens in more detail including the life cycle, sub-types and their identification, pathogenesis, as well as known virulence factors.

1.7.1 *Fusarium* species

Fusarium species commonly are soil-borne and as such are well known as plant pathogens. They can also cause human disease in both immunocompetent individuals (e.g. keratitis, skin or nail infections) and those who are immunocompromised (e.g. bloodstream infections in patients on immunosuppressive chemotherapy for cancer). The genus *Fusarium* incorporates up to 1000 species, predominantly identified based upon the appearance of their spores and colonies. *Fusarium* sp. produces 3 types of spores,[91] as shown in Figure 2.

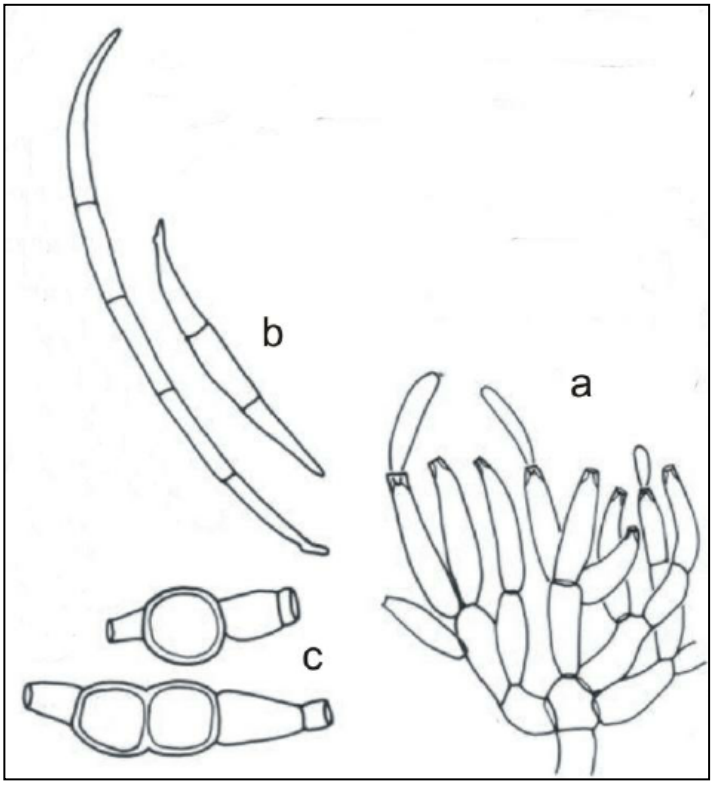


Figure 2: Spore morphology in *Fusarium oxysporum* showing (a) microconidia, (b) macroconidia and (c) chlamydospores.

(Source: Okungbowa, FI, et al, Environmental Research Journal, 2012).[91]

Species of Fusarium that most frequently cause keratitis in South India are *Fusarium solani*, and *Fusarium oxysporum*.[15, 92] Other species that have been reported to cause keratitis include: *F. aquaeductum*, *F. dimerum*, *F. verticilloides* (also known as *F. moniliforme*), *F. nivaleb*, *F. subglutinans*, and *F. ventricosum*.[44] Agricultural workers who are engaged in harvesting of crops using manual methods, may sustain a corneal trauma due to vegetative matter accidentally abrading the ocular surface. If *Fusarium* spores are present on the vegetative matter, they may have a route of entry into the cornea via a corneal abrasion. Once spores enter the cornea, they germinate, begin to develop a germ tube, then elongate into a fungal filament. Pattern recognition receptors, e.g. *Dectin-1*, *TLR2*, *TLR4*, *TLR9*, are involved in initiating the innate immune response in *Fusarium* keratitis, since these receptors show increased gene and protein expression in human epithelial cell-line studies as well as murine models of *Fusarium solani* keratitis.[93, 94] Fungal filaments then continue to grow and branch in corneal tissue. There may be some histologically unique patterns in the morphology of *Fusarium* filaments in biological tissues. Some case reports have described *Fusarium* filaments to develop branches at approximately 90° from the main hyphae.[95] Others have described the presence of spores within corneal tissue in *Fusarium* keratitis (known as adventitious sporulation), as observed in histological sections of tissue obtained at corneal transplantation surgery, although this has not as yet been reported in any *in vivo* confocal microscopy studies of *Fusarium* keratitis.[96] It may therefore be theoretically possible to distinguish *Fusarium* keratitis from *Aspergillus* keratitis using these features, using *in vivo* confocal microscopy imaging.

Fusarium sp. have developed virulence factors that enable them to evade the immune system. The PacC gene in *Fusarium oxysporum* may play a role in allowing the fungus to adhere to and grow successfully within corneal tissue.[97] *Fusarium* sp. can elaborate extracellular proteases that are capable of degrading collagens in the host corneal tissue, thus enabling invasion into deeper stroma.[98] Formation of a biofilm around the fungal mass in the cornea, especially in *Fusarium solani* keratitis, can protect the fungi from the host immune response as well as antifungal treatments.[99] *Fusarium* sp. are also able to generate toxins that could theoretically damage host cells.[100, 101] Prior studies have show some of these toxins (T2-toxin, diacetoxyscirpenol, fusarenone-X) to be toxic to dermal cells.[102] Although some of these toxins (T2-toxin, deoxynivalenol, diacetoxyscirpenol, nivalenol, fusaric acid, moniliformin and

fumonisin B1) are indeed produced by *Fusarium* isolates from human FK, toxin presence was not associated with clinical severity of disease, treatment response or outcome.[100, 101]

1.7.2 *Aspergillus* species

Aspergillus species are among the most frequent causes of FK in India, and often these corneal ulcers have worse clinical outcomes.[65] The main species that cause keratitis are *Aspergillus flavus* and *Aspergillus fumigatus*.[17, 103] Similar to *Fusarium* sp., *Aspergillus* sp. are also soil-borne plant pathogens. *Aspergillus* sp. reproduce asexually through the formation of specialized fungal hyphae called conidiophores that can develop spores (conidospores),as shown in Figure X. These spores are frequently distributed through the air.[17]

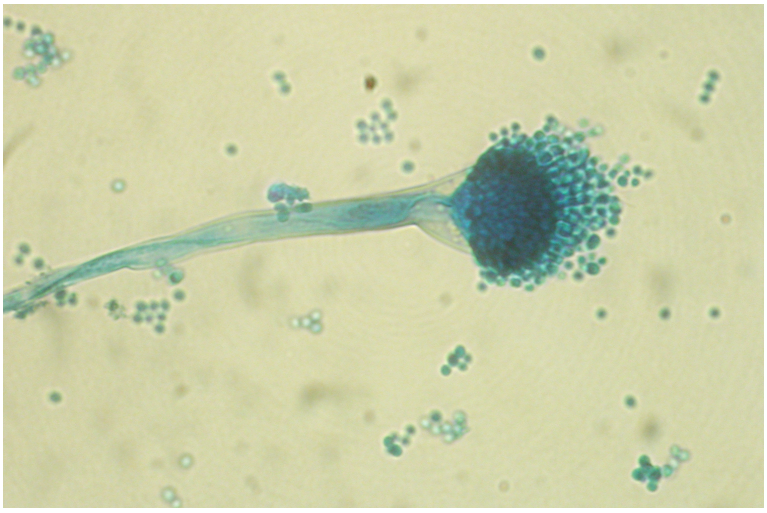


Figure 3: *Aspergillus flavus* hypha that has developed into a conidiophore that bears spores (i.e. conidiospores). Fungus stained with lactophenol cotton blue. Source: Dr. Lalitha, Dept. of Microbiology, Aravind Eye Hospital.

Once the spore germinates and begins to grow a germ tube, molecules on its surface become exposed, that were previously shielded by the hydrophobic spore outer-shell. These molecules, e.g. beta1,3-glucan and alpha-mannose, are able to activate host pattern recognition receptors (e.g. Dectin-1 and Dectin-2), thus initiating an innate immune response in the cornea.[104] Fungal filaments often continue to grow into the cornea, despite triggering of this initial innate response. Chronic, persistent presence of fungal cell wall molecules can dampen the immune response, e.g. beta-glucan can suppress TLR4 responses, and alpha-glucan causes lesser production of IL6 over time when TLR2 and TLR4 are stimulated.[105] Biofilm formation around the fungal hyphal colony in the cornea may also reduce the abililty of the host inflammatory cells to physically reach the fungi in order to

attempt pathogen killing.[106] Mycotoxins (e.g. aflatoxin B1) are produced more often in keratitis-causing strains of *Aspergillus flavus* than environmental strains, however there is toxin production is not specifically associated with worse clinical outcome – larger studies are required to confirm this finding.[107] Proteases (e.g. ALP1) produced by keratitis-causing strains of *Aspergillus* sp. are not only involved in corneal tissue destruction,[98] but may also be able to subvert the host immune response, e.g. through breaking down complement components.[108] Aspergillus fungal filament branching pattern has been examined in histological studies of human corneal tissue, and case reports of *in vivo* confocal microscopy studies of fungal keratitis; these studies have found a branching angle of 45° for *Aspergillus* sp.[95, 109] In addition, dichotomous branching (i.e. apical fungal filament splitting into two branches, as shown in Figure 4), has been associated with *Aspergillus* sp. in histological tissue sections.[110]

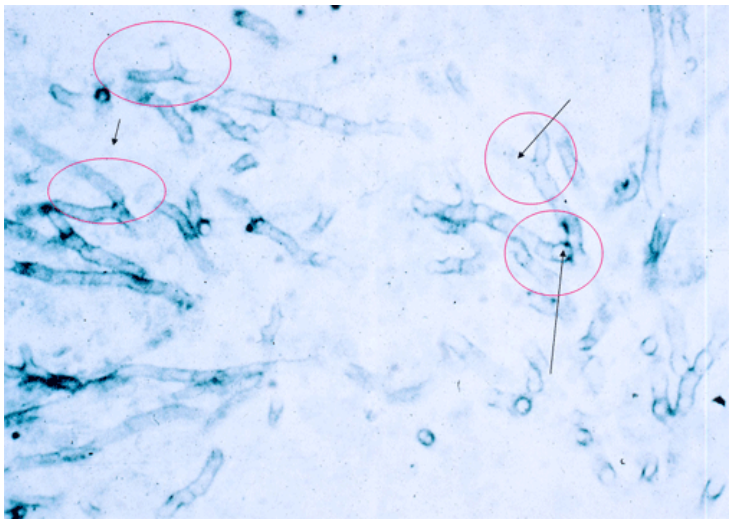


Figure 4: Histological tissue section from pulmonary aspergillosis. Red circles indicate regions of dichotomous branching. (Source: http://www.microbiologybook.org/mycology/mycology-5.htm)[111].

Since *Aspergillus* keratitis often does not respond to maximal medical therapy and therefore may have worse outcomes, there is a need for larger studies to confirm the possibility of identifying *Aspergillus* filaments using their morphology in IVCM images in FK.

1.7.3 *Acanthamoeba* species

Acanthamoeba sp. are free-living parasites that survive in the environment in double-walled cyst form. They are often present in soil as well as water, including domestic tap water. Once in the presence of a food source (e.g. bacteria such as *E. Coli*) *Acanthamoeba* sp. undergo a process of ex-cystment, where the active form of the parasite, the trophozoite, breaks out of the cyst via an opening in one part of the double-wall called the ostiole. In vivo confocal microscopy imaging of *Acanthamoeba* cysts both *in vivo* and *ex vivo* have shown appearances of the cyst to form a double wall with smooth spherical external wall and corrugated internal wall of the cyst (see Figure 5a).[112] At the early stages of ex-cystment as the trophozoite approaches the ostiole, the double walled cyst takes on a "signet ring" appearance.[113] Trophozoites (Fig. 5b) are less frequently seen in IVCM images as they may occur very early on during the course of infection[114] – usually once the patient reaches the cornea clinic and has been started on various antimicrobial agents the trophozoites have undergone encystment.

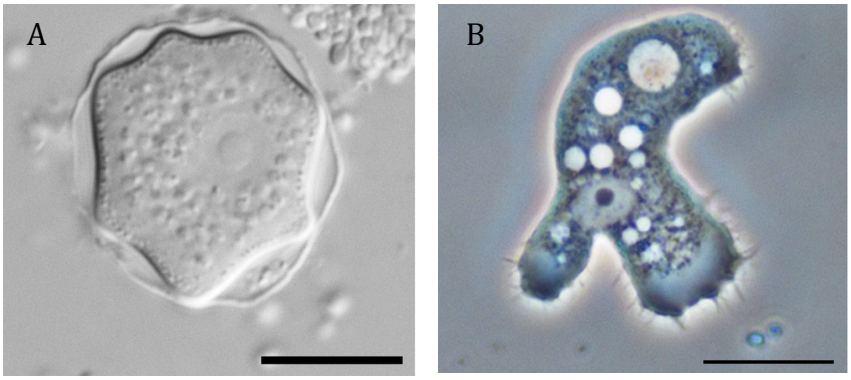


Figure 5 showing (a) an *Acanthamoeba* trophozoite (scale bar 100μm) and (b) a double-walled *Acanthamoeba* cyst (scale bar 10μm). (Source: J. Lorenzo-Morales et al, Parasite, 2015).

Molecular genotyping has identified that three main genotypes are associated with keratitis: T3, T4 (also known as *Acanthamoeba castellani*) and T11.[115] Virulence factors associated with the development of keratitis include the *Acanthamoeba* mannose-binding protein that enables the parasite to bind to corneal epithelial cells and subvert the host immune response by inducing apoptosis in host cells, as well as the production of proteases by *Acanthamoeba* that can also contribute to this direct cytopathic effect on host cells.[116]

1.8 Outcomes in MK: Indian Studies

Once the diagnosis has been made and antimicrobial treatment started, extensive corneal damage can still occur in late-stage MK, resulting in central corneal scarring and sometimes even corneal perforation (as shown in Figure 6).

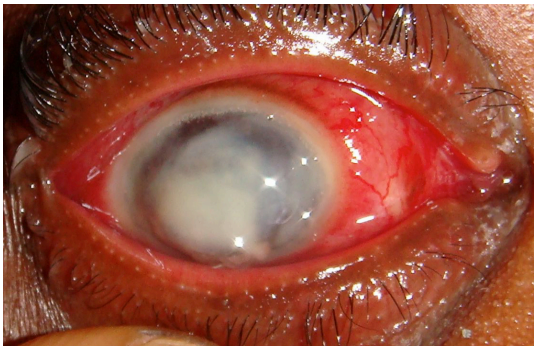


Fig. 6a) Total corneal necrosis due to *Pseudomonas aeruginosa* infection

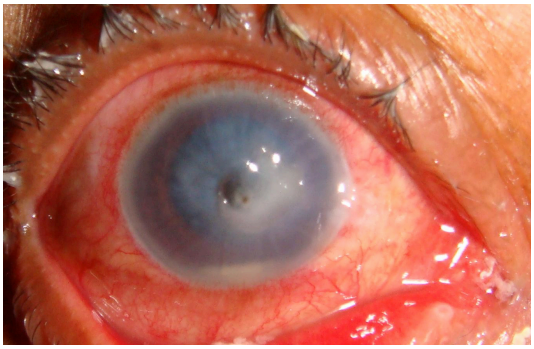


Fig. 6b) Smaller central perforation in fungal keratitis

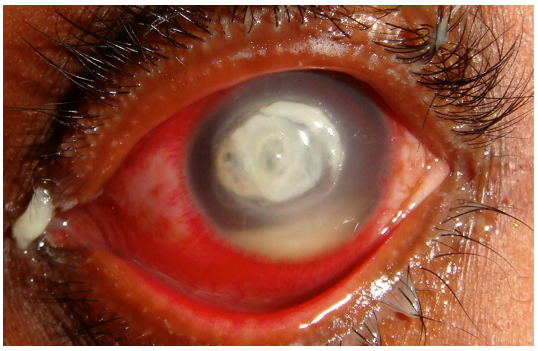


Fig. 6c) Severe keratitis due to *Aspergillus flavus*, with perforation at presentation.

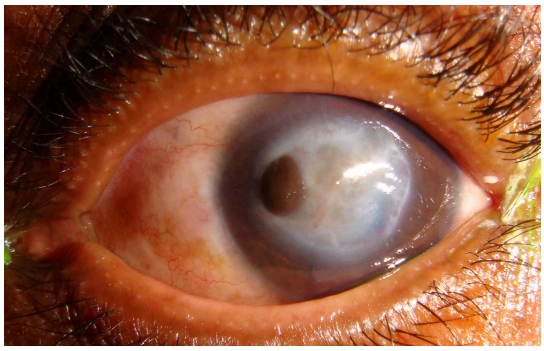


Fig. 6d) Same ulcer as Fig. 6c two weeks later. Corneal perforation has developed, sealed by iris prolapse.

Figure 6: Corneal perforation in severe bacterial and fungal keratitis (Source: Prof. Matthew Burton, KCMC Hospital, Moshi, Tanzania)

Outcomes in FK can be poor, with up to 31% of patients failing to respond to maximum medical therapy.[65] Indian epidemiological studies have reported that final visual acuity can be very low in FK with 35% of patients reported as having best-corrected vision of 6/60 or worse at the end of treatment.[28] In contrast to *Fusarium* keratitis, *Aspergillus* keratitis frequently does not respond as well to intensive natamycin treatment.[117] In severe MK in India, therapeutic penetrating keratoplasty (TPK) was performed in up to 59.4%[42] of FK

patients (often more frequently in *Aspergillus* keratitis[28, 42]), and less frequently in BK, i.e. up to 15.8%[9] of patients (Table 9). Corneal perforation or impending perforation occurred in up to 24%[28] of FK patients, and up to 23.4%[9] of BK patients, often requiring surgical intervention (e.g. corneal glue or TPK), as summarized in Table 9.

Since many patients with severe MK, and especially FK, have poor visual acuity after their course of treatment, or have required surgical intervention for perforation of the cornea, it is increasingly important to understand if there are any potential risk factors that may be associated with these poor outcomes. Very few prospective studies have explored the epidemiological risk factors for clinical outcomes in MK in India (summarized in Table 1). Lalitha *et al* identified the main risk factors associated with worse outcome in FK (i.e. primary treatment failure or perforation) in their South Indian study population to be large infiltrate size at presentation (i.e. >14 mm^2), culture-positivity for *Aspergillus* sp. and presence of hypopyon at the initial visit.[65] Rautaraya *et al* also found that larger ulcer size at the first visit (>25mm^2) was associated with poor outcome in BK, as well as older age and poor presenting visual acuity.[41] Time delay in starting an appropriate antimicrobial (in particular fortified antibiotics for BK) was significantly associated with corneal perforation.[48]

Hence overall, these studies indicate delay in presenting to the ophthalmologist, and therefore more severe MK and inflammation at the start of therapy was linked with worse outcomes, and lack of effective therapy for *Aspergillus* also resulted in more aggressive disease. In view of the very few studies in India that have reported risk factors associated with poor outcomes in severe MK, we have included this as part of the epidemiological analysis within the studies in this PhD.

Table 9: Outcomes in microbial keratitis: Indian studies (abbreviations: LOF=loss of follow-up; TPK=therapeutic penetrating keratoplasty)

First author, (Zone, City, State, Study period)	Observation period	Healed	Final best-corrected visual acuity	Worse	Perforated /Glue	TPK	Graft outcomes	Evisceration	Other
Chowdhary A, 2005[28] (North: New Delhi, 1999-2001)	110 FK pts: mean follow-up 5.5 months (range 2–9 months)	With scar 59% (65/110)	≥20/40 in 53% (58/110); 20/60-20/100 in 16% (17/110); ≤20/200 in 32% (35/110)	Worsened & required TPK 9% (10/110)	Adherent leucoma 41% (45/110), of which Perforated and required TPK: 24% (26/110)	18.8% (36/110; 26 perforated, 10 worse) Aspergillus sp. in 36% (13/36) & Fusarium sp.in 22% (8/36)	FK recurred in 14 (in ≤4 wks of TPK), regraft in 4. Final BCVA after TPK: ≤20/200 in 58% (21/36); 20/100-20/200 in 28% (10/36); 20/80 in 14% (5/36)	7% (8/110)	8% (9/110) secondary glaucoma needing trabeculetomy
Gopinathan U, 2009[9] (South: Hyderabad, Telangana, 1991-2001)	2729 pts; final follow-up ≤3 months	With scar 76% BK (1151/1524); 65% FK (736/1135); 90% AK (63/70)	NM	1.3% BK (19/1524); 1.8% FK (20/1135); 1.4% AK (1/70)	Glue 21% (753/3563); i.e. 23% BK (432/1849); 19% FK (257/1360); 10% AK (9/86); 22% mixed BK/FK (52/236) Adherent leucoma 1.1% BK (17/1524); 0.3% FK (3/1135); 1.4% AK (1/70)	19% (688/3563); i.e. 16% BK (292/1849); 24% FK (321/1360); 3% AK (3/86); 29% mixed BK/FK (68/236)	Failed graft 36% BK (105/292); 32% FK (102/321); 33% AK (1/3) Graft infiltrate 33% BK (98/292); 22% FK (71/321); 0% AK (0/3)	6% (221/3563); i.e. 8% FK (113/1360); 4% BK (75/1849); 3% AK (3/86); 11% mixed BK/FK (27/236)	Significantly more FK needed TPK than all others ($p<0.05$), and glue in BK than all others ($p<0.05$).
Lalitha P, 2006[65] (South: Madurai, Tamil Nadu, 2004)	140 FK, follow-up 4 weeks (LOF n=25)	Healed or slowly healing by final visit 68.6% (79/115)	-	Same, worse, perforated or TPK in 31.3% (36/115)	Perforated in 19% (22/115)	-	-	-	-

Table 9 continued

First author, (Zone, City, State, Study period)	Observa-tion period	Healed	Final best-corrected visual acuity	Worse	Perforated /Glue	TPK	Graft outcomes	Evisceration	Other
Rautaraya B, 2014[41] (East: Bhubaneshwar, Odisha, 2006-10)	303 BK; follow-up 3 weeks (LOF n=34)	healed scar or resolving ulcer in 62.0% (188/303), i.e. in 14.3% in <2 wks	-	Worse 3.3% (10/303); same 6.3% (19/303)	Glue alone 17.5% (53/303); Glue+TPK 3.6% (11/303)	TPK 15.5% (47/303)	Failed graft 64% (30/47)	Evisc 2.6% (8/303)	DCR 2.3% (7/303); orbitotomy 0.3% (1/303); corneal tear repair 0.3% (1/303).
Rautaraya B, 2011[43] (East: Bhubaneshwar, Odisha, 2006-9)	264 FK (incl 45 mixed BK/FK); mean follow-up 43 days (SD 115 days) (LOF n=43)	healed in 42.5% (94/221)	NM	Worse 16/221	Glue 22.6% (50/221)	TPK 52/221	NM	9/221	-
Bandyopadhyay S, 2012[40] (East: Sundarban, West Bengal, 2007-2011)	399 FK: follow-up period not reported	NM	NM	NM	3.3% (13/399) perforated at presentation	NM	NM	NM	-
Nath R, 2011[22] (East: Dibrugarh, Assam, 2007-9)	157 FK alone (+ 27 mixed BK/FK): follow-up period not reported	Healed or improved in 84% (132/157)	NM	Same in 5.7% (9/157)	Perforated in 12.1% (19/157)	NM	NM	NM	2 FK endophthalmitis

Table 9 continued

First author, (Zone, City, State, Study period)	Observation period	Healed	Final best-corrected visual acuity	Worse	Perforated /Glue	TPK	Graft outcomes	Evisceration	Other
Saha S, 2009[42] (East: Kolkata, West Bengal, 2008)	74 culture +ve FK: follow-up period not reported	With scar 40.5% (30/74)	NM	NM	NM	59.4% (44/74) i.e. 36% *Aspergillus* sp. (16/44) & 11% *Fusarium* sp. (5/44). 10 needed TPK at presentation	NM	NM	-

1.9 *In vivo* confocal microscopy (IVCM)

Use of slit lamp examination of the cornea often does not reveal signs of a possible causative organism or signs of improvement/worsening in many cases of MK. The reason for the limited resolution of slit lamp biomicroscopy (i.e. up to 20μm, and 40x magnification) in MK is light scatter from tissue adjacent to the point of interest causing image degradation.[118] The newer imaging modality of confocal microscopy enables a much higher resolution view of the cornea by removing this light scatter in two ways. Firstly, the illumination light source is passed through a small aperture (a slit or pinhole) then focused on to the point of interest by an objective lens. Secondly, only light returning from the point of interest is collected by this objective lens to form the final image (see Figure 7).[119] Since the incident and reflected light are arriving at and returning from the same focal point, this is known as a "confocal" system (developed by Goldmann in 1940, and patented by Marvin Minsky in 1957).[120, 121]

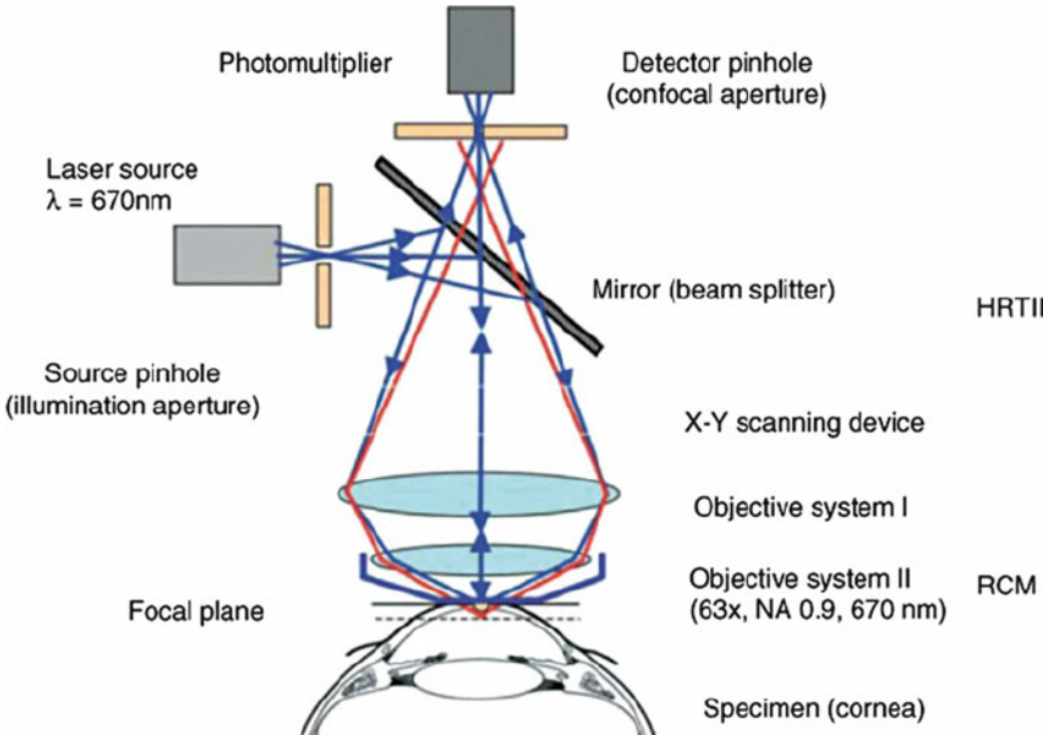


Figure 7: How confocality works in the HRT in vivo confocal microscope

A pinpoint of laser light (formed at the illumination aperture) enters the objective lens systems I & II within the Rostock Corneal Module (RCM) and on to the corneal surface. Light returning only from the focal point of interest, i.e. focused through the same RCM objective lenses, is collected by a photomultiplier at the detector pinhole. Although this diagram is for the HRTII, the HRTIII system uses exactly the same hardware and lens system. (Source: Guthoff R, et al, Clin Experiment Ophthalmol, 2009).[122]

For optimal image resolution in the confocal system, the focal point of the lens has to be very small and this results in a small field of view. Therefore, to image a larger area of the tissue the incident light spot had to be moved in a raster pattern sequentially across the tissue. This was achieved in early confocal microscopes with a Nipkow disc, which had a series of pinholes that were arranged in an Archimedian spiral formation, so that rotation of the disc resulted in shifting of the light spot across the tissue. A second Nipkow disc was used in tandem and light reflected from the point of interest that passed through this second disc was recorded using a video recorder.[119] The Tandem scanning confocal microscope (TSCM) used these specifications, and was the first confocal microscope used to image the *ex vivo* human cadaveric cornea.[123] Using the same technique, Lemp *et al* were able to safely image the living human cornea in 1990.[118]

There were several limitations of the TSCM, including its resolution (~5μm in the X-Y axis) due to light blockade by the pinholes of the Nipkow disc, as well as the inability to perform real-time imaging since processing was required after image acquisition before the image could be observed.[119] The next generation of corneal confocal microscope, the Confoscan (Nidek Technologies, Italy), overcame these limitations by using a brighter light source (white light passed through a slit-aperture, not a pin-hole), and a motorized mirror to rapidly move the light source across the region of interest allowing real-time viewing of the detected image. The current model, Confoscan 4, has a magnification up to 500x with resolution of 0.6 μm laterally (X-Y axis) and 5 microns axially (z-axis) with an image size of 460 x 345 μm (corresponding to 768 x 576 pixels).[124]

More recently, creation of an IVCM with a further improved light source (near infra-red laser, 670nm wavelength) and corneal objective lens (Rostock Corneal Module, RCM, with high numerical aperture of 0.9, allowing 63x magnification), enabled better contrast and higher resolution (both lateral and axial) than the Confoscan 4.[125] The RCM was develop by Guthoff *et al* in Rostock University, Germany, however no optical ray diagrams are available in the medical literature to show in detail the path of a ray of light through the objective lenses within the RCM. The best ray diagram available for the HRT IVCM is shown in Figure 1. The Heidelberg Retinal Tomograph (HRT3) IVCM with RCM allows a lateral resolution of up to 1μm (X-Y axis), up to 2μm axially (Z-axis) with 800x magnification.[112] The laser spot is moved in a raster pattern to generate an image measuring 400μm (in the X-axis) by 400μm

(in the Y-axis)(corresponding to 384 x 384 pixels). The RCM also incorporates a motor that allows automated focusing of the RCM lens so that it is possible to automatically acquire a set of 40 sequential 400 μm x 400 μm images, covering a total depth of 80μm in the Z-axis (i.e. each imaging focused 2μm deeper into the cornea than the preceding image). The HRT3/RCM system can therefore allow image capture in 3 ways: section scan (i.e. single image measuring 400 μm x 400 μm in the X-Y axis); volume scan (i.e. z-stack of 40 images measuring 400μm (X-axis) x 400μm (Y-axis) x 2μm (Z-axis); sequence scan (i.e. continuous video capture of the single image measuring 400 μm x 400 μm in the X-Y axis but captured continuously at between 5 to 30 frames per second, chosen by the operator, at a focus-depth specified by the user). Volume scan is a rapid method to image deeply into the cornea and so is used by many studies of IVCM in corneal diseases involving the stroma, e.g. MK. The HRT3 software allows the operator to reset the corneal depth information to zero at the start of imaging at the most superficial corneal layer, and depth information is linked to the focus position of the RCM lens, hence each image captured has depth information allocated to it. The HRT3 software has limited options for image analysis, allowing the operator to be able to select an image, mark a region of interest and then manually mark cells, thus allowing a cell count per mm^2 within this region. Thus most researchers have exported HRT3 IVCM images for analysis in external software, or have written their own software for image analysis.

Images of the cornea using the HRT3/RCM IVCM are able to show intracellular structures, e.g. nuclei and intra-cytoplasmic granules within activated keratocytes, and pathogen features such as double-walled *Acanthamoeba* cysts and fungal hyphae in greater detail than with the Confoscan 4.[126]

Although the HRT3/RCM IVCM provides high resolution images of the cornea, it does have limitations. The operator uses manual focusing of the RCM and can view the image generated on a computer screen, before deciding to capture an image. But since this image is viewed by the operator at a cellular level with no fixed landmarks being available in the cornea to allow orientation (such as optic disc or macula in the retina), at present it is not possible to know the exact X-Y-Z axis location of this image within each layer of the cornea, in order to be able to accurately re-image the same region of the cornea in the same patient again (i.e. spatial registration of the image) over time (i.e. temporal registration of serial images).

1.10 IVCM of the Normal Cornea

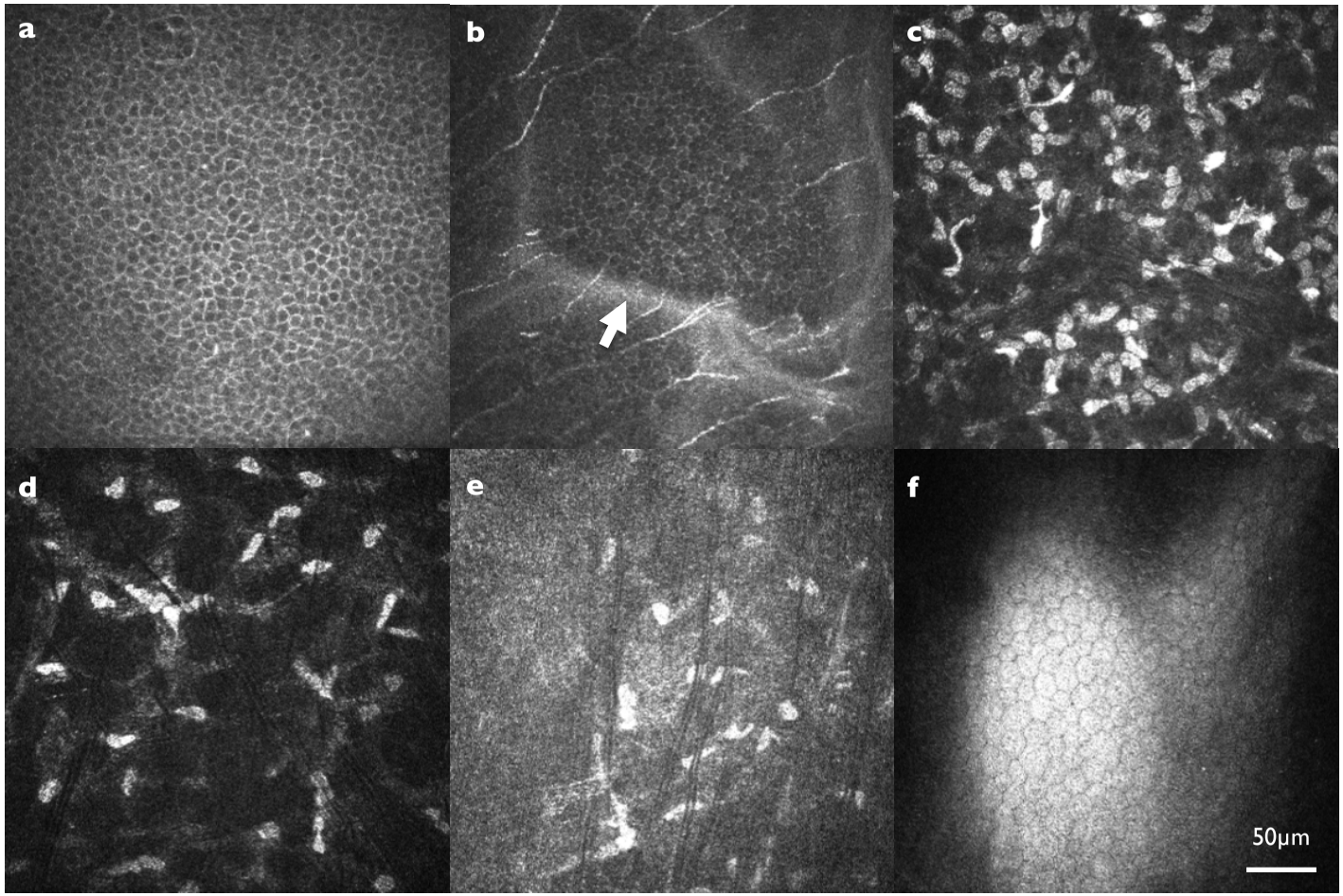
HRT3/RCM provides *en face* images of the living cornea at the cellular level. Refractive interfaces between or within cells or other structures in the cornea (e.g. fungal hyphae) can be observed with IVCM. Figure 8 shows the images obtained with this technique in different layers of the normal human cornea. Epithelial cells within the normal cornea appear within IVCM images with a dark centre, barely visible nucleus and very brightly reflective border, with neighbouring cells closely apposed to each other. Epithelial cell diameter decreases in the anterior to posterior direction, with smallest cells observed in the penultimate wing cell layer (Fig. 8a) and basal layer, forming the highest cell density in these layers.

Immediately beneath the basal epithelial layer, the sub-basal nerve fibre layer can be observed with IVCM as highly reflective fine linear, branching structures (shown in Fig. 8b) that form a central whorl near the corneal apex and emanate outwards toward the peripheral cornea. A small population of antigen presenting cells (Langerhans cells) resides in the central cornea and peri-limbal peripheral cornea in this sub-basal region and these are seen as bright dendritiform cells in IVCM images. Bowman's membrane is seen with HRT3 IVCM as curvilinear lamellae (with indistinct margins, shown by arrow in Fig. 8b) passing beneath the basal epithelial cell layer.

HRT3/RCM imaging of the corneal stroma shows the presence of ovoid hyper-reflective keratocyte nuclei, with barely visible cellular processes in the normal cornea. The keratocyte density is highest in the anterior stroma (Fig. 8c), decreasing towards posterior stroma (Fig. 8d), but with a slight increase in cell density immediately before Descemet's membrane. Descemet's membrane itself appears as a homogenous structure on IVCM imaging with a ground-glass appearance (Fig. 8e). Finally, normal endothelial cells can be seen as a monolayer of cells with bright cytoplasm but dark borders (Fig. 8f). Occasionally, endothelial cell nuclei appear as dark regions in the centre of each cell in the IVCM image.

Figure 8: HRT3 In vivo confocal microscope images of the normal cornea

Images show (a) the wing cell layer of the epithelium; (b) the basal cell layer of epithelial cells interspersed with corneal nerves (linear branching structures) and collagen lamellae of Bowman's membrane (arrow); (c) keratocytes of the anterior stroma; (d) keratocytes of the posterior stroma; (e) Descemet's membrane with posterior keratocytes nuclei visible through this layer; (f) endothelial cell layer. Each image measures 400 x 400 µm.



1.11 IVCM in the Diagnosis of Microbial Keratitis

IVCM can be used to detect organisms within the cornea if their size is greater than the limit of resolution of the microscope (i.e. $\geq$1μm for the HRT3/RCM IVCM). In particular, fungal hyphae and *Acanthamoeba* cysts are readily seen with IVCM due to their highly reflective outer wall.[109, 127]

Fungal Hyphal Morphology in IVCM Images

In the living human cornea, IVCM was first used to image fungal keratitis in 1997.[109] Using the tandem-scanning confocal microscope the authors described the appearance of the *Aspergillus* sp. as highly reflective linear branching structures, measuring up to 6 μm in diameter and 200-400μm in length, with new branches forming at an angle of 45˚ from the main branch.[109] Since then, several other case reports also documented similar dimensions of *Aspergillus* hyphae within the human cornea of patients with FK.[95, 109, 128]

Fusarium sp. filaments have been described to have comparable dimensions in IVCM imaging with a diameter of 3-5μm and length of 200-300μm.[95] In contrast to *Aspergillus* sp., the branching angle of *Fusarium* hyphae as observed in IVCM images has been measured as 90˚.[95] However, evidence from histopathological examination of fungal growth in tissue shows that acute angle branching (i.e. $\leq$45˚) may occur in both species.[110]

Many factors are known to affect branching angle in filamentous fungi, including gene mutations in the microtubule assembly pathway[129], presence of a hyperosmotic environment[130], tendency for new hyphal tips to grow away from adjacent hyphae [130] (known as negative autotropism[131]), and alterations in surface topography of the tissue.[132] Also, exposure to antifungal agents that block fungal cell wall synthesis as part of their mechanism of action can induce increased branching frequency in *Aspergillus sp.* hyphae. [133-135]

Since clinical outcomes for *Aspergillus* ulcers can be much worse than for *Fusarium* ulcers[65], it would be advantageous to be able to identify fungal species early on in the infective process, especially with a non-invasive tool such as IVCM that could be used in clinic at the first visit. There is currently a lack of evidence in the literature, in the form of studies with large patient numbers, to confirm whether branching angle is indeed significantly different between

Fusarium sp. and *Aspergillus* sp. in human FK and therefore whether this parameter can be used to differentiate between the two species.

Clinical trial data suggests that natamycin treatment should be the first line therapy for filamentous fungal keratitis, and is particularly effective against *Fusarium* sp., although less so for *Aspergillus* sp.[51] Topical voriconazole used alone is not more effective than natamycin against either *Fusarium* or *Aspergillus* sp[51], however when used in oral form in addition to natamycin it may improve outcomes for *Fusarium* keratitis only.[136] As such, knowledge of the species of fungus causing MK early on would allow for intensive monitoring of *Aspergillus* keratitis and a low threshold for surgical intervention (i.e. TPK) in worsening ulcers, or use of natamycin +/- oral voriconazole for *Fusarium* keratitis.

False positives and false negatives in the detection of fungi in IVCM images

Other structures in the healthy as well as diseased cornea can present as linear, branching structures in IVCM images, and as such be graded as false positives for fungi. Corneal nerves in the basal nerve plexus can appear similar to fungi, and also, long cellular processes of dendritiform cells, if captured in an IVCM without the cell body, can also appear as branching structures that mimic fungi. Experienced observers often are able to accurately detect fungi apart from these other structures since they have experience of the morphological differences that are unique to fungi rather than these other structures,[137, 138] however inexperienced observers may falsely grade these structures as positive for fungal filaments when they are actually not fungi.[139] False negatives can occur if the region of the cornea containing the pathogen is not imaged by the confocal microscopist (e.g. inexperienced operator), or if the fungal filaments are fine so are not obvious in the IVCM images, or if the IVCM grader has not have enough experience to detect less obvious filaments (i.e. inexperienced grader).[137, 140]

IVCM Diagnostic Accuracy for Fungal Keratitis

Many case reports have shown the utility of IVCM in the detection of fungal filaments in the cornea in patients presenting with microbial keratitis (summarized by Labbe *et al*, 2009[112]). However, few studies have sought to formally investigate the sensitivity and specificity of the technique (studies summarised in Table 10). Kanavi *et al*, used the Confoscan 3.0 (Nidek Technologies, Italy) to image the cornea in 133 patients presenting to the eye clinic in Iran with microbial keratitis.[141] Using microbiological culture and light microscopy (of corneal

scrapes and/or contact lens cases) as the reference standard, the authors found a sensitivity of 94% and specificity of 78% for detection of fungal filaments in IVCM images.[141] It is not stated in this paper whether the study was performed as a retrospective casenote review or a prospective study, and also the method of IVCM grading, i.e. level of experience of the grader, and whether the grader was masked to clinical features of the ulcer, each of which can have an impact on the final diagnostic accuracy of the test.[137]

Vaddavalli *et al*, took these factors into account when conducting a prospective study with the Confoscan 3.0 in 146 consecutive patients presenting with MK in South India, in a region with high incidence of fungal keratitis.[142] Two IVCM graders who were masked to the history and clinical features of each case graded the IVCM images for presence/absence of fungi, thus allowing for inter-observer agreement to be measured. The grading process was repeated two months later to allow intra-observer variability to be calculated. Overall, sensitivity for detection of fungal hyphae in IVCM images was found to be 89.2% (95% CI, 83–95.5) and specificity was 92.7% (95% CI, 85.9–99.6).[142] The inter-observer agreement was moderate (kappa score 0.6; phi 0.617), which the authors felt was due to differing levels of experience of the two graders. The intra-observer agreement was considered to be good (kappa 0.795; phi 0.807).

Table 10: Sensitivity and Specificity of *In Vivo* Confocal Microscopy to Detect Fungal Keratitis in settings with Low/High Incidence. (PPV=positive predictive value; NPV=negative predictive value; NM=not mentioned)

Author	Country	Incid-ence FK*	Main fungi	IVCM	Sensitivity FK	Specificity FK	PPV	NPV	Inter-grader agree-ment	Intra-grader agree-ment
Vaddavalli P, et al, 2011	Hydera bad, South India	62.8% of all MK (93/148)	*Fusarium* sp *Aspergillus* sp	Confos can 3	89.2% (95% CI: 83–95.5)	92.7% (95% CI: 85.9–99.6)	95.4% (95% CI: 91–99.8)	83.6% (95% CI: 74.3–92.9)	Kappa=0.6 (phi=0.617)	Kappa=0.79 5 (phi=0.807)
Kanavi MR, et al, 2007	Tehran, Iran	12.0% of all MK (16/133)	NM	Confos can 3	94%	78%	55.6%	97.7%	One grader only	NM
Kheirkhah A, et al 2017	Boston, USA	46.7% (21 images of FK, only 11 culture +ve for FK & 24 images culture +ve BK)	Filamento us fungi (yeast excluded): species NM	HRT3 (sequen ce scan mode)	Experienced grader 71.4%; inexperienced 38.1% & 47.6%	Experienced 91.7% & 87.5%; inexperienced 75% & 100%	Experienc ed 88.2% & 83.3%; inexperien ced 57.1% & 100%	Experienced 78.6% & 77.8%; inexperience d 58.1% & 68.6%	Experienced κ=0.77, P<0.001; inexperience d κ=0.51, P<0.001	Experienced κ=0.86, P<0.001 & κ=0.91, P<0.001; inexperience d NM

*Culture and/or light microscopy positive for fungal filaments

Acanthamoeba Morphology on IVCM

Acanthamoeba cysts were first observed in the living human cornea in 1994 using the scanning-slit confocal microscope.[143] Since then, IVCM imaging of *Acanthamoeba* cysts has shown various different morphologies:

1) Double-walled cyst or "bright spot" sign

The classical appearance of the double-walled *Acanthamoeba* cyst, as observed with IVCM, measures between 10-20 μm in diameter (as measured within both *in vivo* and *ex vivo* HRT3 IVCM images of cysts)[122, 144]. Cysts can occur anywhere in the epithelium or stroma and can sometimes be observed to align themselves along corneal nerves during peri-neuritis, or in lines or clusters in Bowman's membrane.[143, 145, 146] Depending on the amount of incident light and angle of illumination, cysts can appear to be uniformly hyper-reflective, resembling a "bright spot" with no distinct double-wall visible.[147]

2) Signet-ring

The double-walled cyst displays a bright highly reflective spot associated with the outer wall, resembling a signet ring.[147, 148]

3) Trophozoite

As *Acanthamoebae* ex-cyst, they form a larger trophozoite phenotype, characterized in light microscopy imaging by intra-cellular vacuoles and acanthopodia at the margins of the trophozoite, as well as absence of the cell wall that is present in cysts.[144] A wide variety of morphologies have been described for the *Acanthamoeba* trophozoite in IVCM imaging, such as hyper-reflective objects measuring up to 100 μm in diameter with a surrounding zone of hypo-reflectivity, occasionally with "pseudopodia" or vacuoles detected by some graders within IVCM images.[140, 141, 149] Kobayashi *et al* compared *ex vivo* imaging of *Acanthamoeba* trophozoites (isolated from AK patients) performed with HRT2 confocal microscope versus light microscopy.[144] The authors found an amorphous appearance of the trophozoites with HRT2 imaging, and also described the difficulty in distinguishing trophozoites from other corneal pathology within *in vivo* images of AK.[144] This variable phenotype in IVCM can reduce the ability of IVCM graders to be able to reliably and reproducibly classify the trophozoite structure in IVCM images of AK images. Since the *Acanthamoeba* cyst wall is hyper-reflective and more easily detectable in HRT IVCM imaging, the "bright spot" or "double-walled cyst" appearance of cysts is much more frequently used to make the diagnosis of AK in IVCM images.[113]

False positives and false negatives in the detection of Acanthamoeba sp. using IVCM images

Although *Acanthamoeba* cysts in particular have a consistent morphology in IVCM imaging that can be used to make the diagnosis, the are other structures that have a similar size, shape and reflectivity in IVCM images that can create a false positive grade by the IVCM grader. In particular, inflammatory cells (e.g. neutrophils) have a circular shape with similar diameter to Acanthamoeba cysts (10-20 microns) and similar "bright spot"-type reflectivity and so these cells are frequently falsely diagnosed as Acanthamoeba cysts in IVCM images.[122, 137] False negatives (i.e. microbiologically positive corneal scrape for *Acanthamoeba*, but no cysts seen on IVCM images) usually occur due to inadequate imaging, so that the images reviewed by the grader do not contain any cysts as the region of the cornea containing the cysts was not imaged. This usually occurs if the patient is not co-operative with the IVCM imaging procedure (i.e. very photophobic or not able to keep the eye still for the time required to do the scan), or if the confocal microscopist is not experienced in imaging the cornea and so has not systematically imaged the whole corneal ulcer.[140]

IVCM Diagnostic Accuracy for Detection of Acanthamoeba sp.

The main large studies that have assessed the sensitivity and specificity of IVCM in the detection of *Acanthamoeba* sp. in MK have used the Confoscan IVCM (Table 11).[141, 142, 150] Tu *et al* retrospectively identified IVCM images from 125 patients with clinically suspected AK who had also been imaged with the Confoscan 2.0 or 3.0.[150] With a reference standard of culture-positivity alone, the sensitivity was 92.9% (76.5%–99.1%) with specificity 77.3% (67.7%–85.2%). When using culture or smear or corneal tissue positivity as the reference standard, the sensitivity reduced to 90.9% (78.3%–97.5%), but specificity increased to 90.1% (81.5%–95.6%). Finally, when including only cases that showed clinical resolution after treatment for *Acanthamoeba* sp. and were positive for *Acanthamoeba* sp. in either culture, smear or corneal tissue pathology, the sensitivity reduced to 90.6% (79.3%–96.9%), but specificity greatly increased to 100.0% (95.0%–100.0%).[150]

Kanavi *et al* used the Confoscan 3.0 with a reference standard of culture and/or smear-positivity for *Acanthamoeba* sp. in their series of 133 patients with clinically suspected MK, 15 of whom were diagnosed with AK by the reference standard.[141] They found a sensitivity of 100% and specificity of 84% for the diagnosis of AK from the IVCM images. Vaddavalli *et al* found a sensitivity of 88.3% (95% CI: 82.2–94.5) and specificity of 91.1% (95% CI: 82.8 –99.4) for observing AK in Confoscan 3.0 IVCM images in their prospective study of 148 patients with clinically suspected MK, of whom 10 were reference standard positive for AK (i.e. culture and/or smear positive).[142]

HRT3/RCM offers a higher resolution method of imaging the cornea compared to Confoscan IVCM, and as such may allow for improved detection of fungal filaments and *Acanthamoeba* sp. However, to the best of our knowledge, there are no large-scale prospective studies that have sought to identify its sensitivity and specificity for the diagnosis of FK or AK.

Table 11: Sensitivity and Specificity of *In Vivo* Confocal Microscopy to Detect Acanthamoeba Keratitis in Settings with Low/High Incidence (PPV=positive predictive value; NPV=negative predictive value; NM=not mentioned)

Author	Country	Incid-ence*	IVCM	Sensitivity AK	Specificity AK	PPV	NPV	Inter-grader agreement	Intra-grader agreement
Prospective studies enrolling all MK patients									
Vaddavalli P, et al, 2011	Hyderabad, South India	6.7% of all MK (10/148)	Confoscan 3	80.0 % (95% CI: 55.2–100.0)	100% (95% CI: 97.8–100.0)	100% (95% CI: 62.6–100)	98.6% (95% CI: 96.6–100.0)	Kappa=0.6 (phi=0.617)	Kappa=0.795 (phi=0.807)
Kanavi MR, et al, 2007	Tehran, Iran	11.3% of all MK (15/133)	Confoscan 3	100%	84%	65.2%	100%	One grader only	NM
Retrospective studies with selected MK cases									
Tu E, et al, 2008	Chicago, USA	42.4% of only atypical keratitis pts (53/125)	Confoscan 2 & 3	90.9 (78.3–97.5)	90.1 (81.5–95.6)	83.3 (69.8–92.5)	94.8 (87.2–98.6)	NM	NM
Kheirkhah A, et al 2018	Boston, USA	45.2% i.e. 28 culture +ve AK images selected plus 34 culture +ve BK images selected	HRT3 (sequence scan mode)	Experienced graders: 69.7% ± 2.5%; inexperienced 59.0% ± 7.6%	Experienced 97.1% ± 4.2% & inexperienced 92.7% ± 10.4%	NM	NM	Experienced κ=0.60, P<0.001; inexperienced κ=0.48, P<0.001	NM

*Culture and/or light microscopy positive for *Acanthamoeba* cysts from corneal scrapes (and corneal biopsy results in Tu E, et al

Limitations of IVCM in the diagnosis of MK

Interpretation of IVCM images is entirely based upon recognition of morphological features that are characteristic of the pathogen, and so the studies described above have relied upon IVCM graders to distinguish the pathogen from other corneal structures in MK. This can become difficult if other structures are present within the IVCM image that may resemble the pathogen. For example, leucocytes that have infiltrated into the inflamed cornea in MK are of similar shape, size and reflectivity to *Acanthamoeba* cysts.[122, 137] In the case of fungal filaments, other slender linear branching structures may be present in the cornea in MK, such as cornea nerve fibres in the sub-basal plexus (although the sub-basal corneal nerve plexus in acute MK as observed with HRT3 IVCM is often very diminished in comparison with normal eyes[151]) or the long cellular processes of dendritiform cells (i.e. where the cell body may not be visible within the same IVCM image). In these cases, the IVCM grader's past experience of interpreting MK images becomes important for accurate diagnosis. Hau *et al* recognized that as grader experience increases, then diagnostic accuracy also increases for detection of *Acanthamoeba* and fungal elements in HRT IVCM images of MK.[137] Therefore, there may be some inter-grader variability depending upon the experience of graders used in IVCM studies. Vaddavalli *et al* showed that 2 experienced graders had a very high inter-grader and intra-grader agreement when assessing the presence of fungi or *Acanthamoeba* in confoscan IVCM images of MK.[142] However, no authors have as yet published inter or intra-grader agreement figures for prospective studies using the HRT IVCM in MK to date.

1.12 IVCM to assess the fungal response to treatment

In many cases of MK, especially those caused by fungi, a slow clinical response after the commencement of antimicrobial therapy can pose a problem in management decisions. Slit lamp examination alone may not show any change in size of the ulcer, resulting in ambiguity as to whether there is a response to the treatment or not. In FK, IVCM has been used in a small number of studies to sequentially examine fungal hyphae in the cornea during the course of antifungal treatment; in TSCM or Confoscan IVCM images, fungal hyphae have been observed to fragment, become thinner or to form smaller clumps then eventually disappear with effective antifungal treatment.[109, 152-156] In one of these studies, Winchester *et al* observed *Aspergillus* hyphae break into smaller fragments four days after commencing topical natamycin and oral ketoconazole therapy in a patient with FK imaged using TSCM

IVCM. A further 12 days later, these fragments had formed into "ovoid masses", which then disappeared after another nine days of antifungal treatment.[109]

Very few studies have investigated the utility of IVCM in monitoring clinical progress in FK in a larger number of patients. Shi *et al* followed 110 FK patients over the course of their treatment and monitored fungal hyphae changes and inflammatory cell counts using weekly confoscan IVCM imaging of the centre and margins of the ulcer.[153] Although the authors describe both an increased quantity of inflammatory cells at the ulcer margins as well as an increased amount of fungal filaments in patients with clinically worsening ulcers, they do not quantitate these findings in any way in their reported findings.[153] However, histologically, it has been shown that the amount of inflammatory cell infiltrate is inversely proportional to the fungal hyphal load in FK corneal ulcer tissue[157], therefore the pattern of inflammatory response in the tissue may indeed be prognostically useful. As such, these prior TSCM and confoscan scan studies highlight the possibility of using cellular features visible in IVCM such as morphology of fungal filaments or the inflammatory cell infiltrate to assess clinical response to treatment, however to date no prospective studies have utilized the higher resolution HRT3 IVCM to prospectively follow FK patients over time to quantitate specific IVCM features that correlate with clinical outcome or response to treatment.

1.13 IVCM imaging to assess host response in MK

Many studies have been conducted to quantitatively assess IVCM morphological features after laser refractive surgery, corneal graft surgery, collagen cross-linking or other corneal diseases (reviewed by Hovakimyan *et al*, Patel *et al* and Labbe *et al*[112, 126, 158]) however very few studies have assessed these features in MK. Morphological changes in keratocytes, inflammatory cells, and extracellular changes observed in the corneal epithelium or stroma have been described, with some of these being associated with clinical outcome. Specifically, presence of the following features have been assessed previously in IVCM images of corneal disease:

1) ***"Stellate" interconnected cellular appearance in stroma, nuclei visible***

   In HRT3 IVCM images of injured or inflamed corneal tissue, cells in the stroma appear as bright broad cellular processes with present/absent bright ovoid nuclei (as shown in Figure 2 in Chapter 6). These cells have been postulated to be the resident keratocytes, that respond to injury/disease by becoming activated to a more fibroblast-like state.[126] It may be that keratocyte cellular processes become visible with IVCM because of

reduction in corneal crystallin production and/or the higher corneal water content that occurs in stromal oedema, both of which could contribute to a change in refractive index.[126, 159, 160] This stellate cellular appearance in IVCM images of the corneal stroma was initially described after PRK, but since then has been observed in many corneal diseases including in bacterial and fungal keratitis.[126, 161] One study has even proposed this specific IVCM appearance might be a predictor for corneal graft rejection.[162]

2) *"Stellate" interconnected cells in stroma, no nuclei visible*

The IVCM appearance of apoptotic keratocytes is characterized by a network of bright, interconnected keratocyte cellular processes similar to the stellate activated keratocytes but with one major difference – an absence of any visible nuclei; occasionally highly reflective granular deposits can be observed within the keratocyte cell body or processes.[126] Histological studies of fungal and *Acanthamoeba* keratitis tissue removed at the time of therapeutic penetrating keratoplasty also showed evidence of apoptotic keratocytes with loss of keratocyte nuclei and TUNEL-positivity in the remaining keratocyte nuclei (TUNEL stains fragmented DNA in apoptotic cells).[163] Electron microscopic examination of apoptotic keratocytes also showed fragmented nuclei, and presence of "apoptotic bodies" within the cellular processes of the keratocyte.[159] Although this IVCM appearance of apoptotic keratocytes has not formally been reported in microbial keratitis, it has been described post-PRK,[164] in Fuch's endothelial dystrophy, after acute glaucoma with corneal oedema, in weeks 1-4 post-CXL[126].

3) *"Spindle-shaped" intracellular opacities*

These cells appear with IVCM as linear bright spindle-like opacities in the corneal stroma. The microscopic appearance of these linear opacities correlates with stress fibres, as seen by phalloidin staining of filamentous actin in histological studies of the injured cornea. Stress fibres usually appear within activated keratocytes by 7 days post-injury, often in a random orientation in the centre of the wound.[165] This configuration changes over time with stress fibres moving to become oriented with the long axis of the wound.[165] "Spindle-shaped" appearances in IVCM images have been reported in multiple corneal conditions including bacterial and fungal keratitis, corneal cross linking, and chemical injuries[126], as well as after PRK[164] or LASIK[166].

4) *Dendritiform cells*

An increase in the density of centrally located dendritiform cells beneath the basal epithelial layer has been noted in IVCM images of microbial keratitis, with *Acanthamoeba*

infection showing the highest dendritiform cell density, followed by fungal and then bacterial ulcers. The dendritiform cells were also increased in size with more dendritic processes per cell in microbial keratitis compared to normal eyes, in the IVCM images.[167] Without direct comparison of the same tissue using IVCM and then immunohistochemistry, it can be difficult to be certain of the specific cell types that constitute these dendritiform cells. Some studies have found postulated that dendritic cells or macrophages resident within the cornea can produce a dendritiform appearance,[168] and others report that bone-marrow derived cells that have migrated into the cornea during injury/inflammation can also take on this phenotype.[169]

5) Inflammatory cells

The presence of an inflammatory cell infiltrate can be observed in IVCM images of BK, FK and AK.[122, 137, 155] Mouse models comparing HRT3 IVCM images obtained *in vivo* with *ex-vivo* immunohistochemical staining of the same tissue have confirmed that neutrophils can form a honeycomb distribution along keratocytes in the cornea within hours of a corneal abrasion injury.[170] Also, monitoring the number of inflammatory cells or their disappearance has been suggested by two authors as a method of assessing the response to therapy, particularly in fungal keratitis.[155] Serial HRT2 IVCM imaging of the cornea in canine fungal keratitis (caused by *Fusarium* spp., *Candida albicans* and *Malassezia* spp.), showed progressive reduction in inflammatory cell numbers that paralleled clinical resolution.[155] Shi *et al*, followed 121 patients presenting with FK (predominantly culture-positive for *Fusarium* sp. in 70%) with repeated confoscan IVCM imaging after the start of antifungal therapy.[153] They used the inflammatory cell infiltrate as a parameter to guide further therapy: if the infiltrate increased they performed penetrating keratoplasty, or if it decreased they tapered and reduced the frequency of topical non-steroidal anti-inflammatory eyedrop instillation. In this paper, however, the authors do not describe their quantitative method for enumerating the amount of inflammatory cell infiltrate.

6) Extracellular Changes: Epithelial Bullae, Stromal Bullae & Scarring

The appearance of corneal epithelial and stromal bullae or scarring, as detected in IVCM imaging, have been described in Fuch's endothelial dystrophy.[171] The epithelial bullae observed in IVCM images completely resolved in parallel with resolution of corneal oedema following endothelial keratoplasy. Scar tissue forms in the corneal stroma during the resolution phase of microbial keratitis, and can be visualized in IVCM images.[122] Various IVCM morphologies of stromal scar tissue have been described, varying from

broad bands of homogenous bright reflectivity through to wispy networks of fibrotic scar tissue in bullous configurations.[122, 171]

Identification of the above IVCM morphological features serially over time could therefore potentially be correlated with clinical outcome in MK. However, these morphological features have not as yet been formally assessed in a prospective cohort of MK patients using HRT3 IVCM imaging. Inter and intra-grader agreement has only been assessed in very few studies, and so further research is required to explored these as well as the types of images that create false-positives or false-negative results with the higher resolution HRT3 IVCM.

1.14 The Host Immune/Inflammatory Response in MK

Most of the previous published studies on the host immune and inflammatory responses in bacterial and fungal keratitis have focused only on specific molecules that were up/downregulated in their gene expression or protein production in either animal models of disease, or in human corneal tissue (obtained from surface scrapings in early disease, or tissue excised at the time of corneal transplantation surgery in late stage disease (reviewed in [172-174]). Overall, these studies have offered an insight into a few of the biological pathways involved in corneal ulceration, and some of the causes of tissue destruction in later disease.

Protection at the ocular surface against microbial infection

Multiple mechanisms exist at the ocular surface that are known to be able to prevent bacterial or fungal infection of the cornea. The normal tear film provides a first-line of defense against pathogens, and contains antimicrobial peptides (e.g. lysozyme, LYZ; lipocalin, NGAL; lacritin; cystatins; beta-defensins, DEFB; peptidase inhibitor 3, PI3[175]), surfactant proteins (e.g. surfactant-D), antibodies (secretory immunoglobulin A), complement factors and mucins.[176] However, prior work has shown that these proteins may not be fully effective against the pathogens that cause human MK. In BK, *Streptococcus pneumoniae* may be able to survive the cell membrane-lysing activity of lysozyme due to production of a protective polysaccharide capsule.[177] Although, surfactant-D may be able to reduce the number of *Pseudomonas aeruginosa* bacteria that enter the corneal epithelium (as shown in corneal epithelial cell culture and mouse models of disease[178]), exposure of *Pseudomonas aeruginosa* to whole human tear fluid can induce production of pathogenic molecules (e.g. components of the type-III secretion system that allows injection of bacterial toxins into the host cell[179,

[180]) or release of bacterial products that are toxic to human corneal epithelial cells (e.g. bacterial outer membrane vesicles[181]). Membrane-bound mucins promote the barrier effect of the corneal epithelium by anchoring antimicrobial peptides in the tear-film to the corneal epithelial surface.[176] Soluble mucins can bind pathogens in the tear-film and reduce their ability to adhere to the cornea.[182] However, pathogenic strains of *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* are able to break down mucins, unlike non-pathogenic strains of these bacteria, therefore aiding their ability to invade the corneal epithelium.[183, 184]

In FK, compared to normal tears, tear fluid from patients with *Fusarium* and *Aspergillus* culture-positive FK (obtained using capillary tubes at the globe/lower eyelid) had a lower quantity of antimicrobial lacritin, lipocalin and cystatins, especially in the early stage of infection (i.e. <7 days from symptom onset).[185], Higher levels of both complement components as well as inhibitors of complement activity (e.g. complement factor H) were found in tears of patients with *Aspergillus flavus* keratitis compared to tears from normal study participants.[186]

Breaching the corneal epithelium

Once a pathogen has overcome the antimicrobial properties of the tear-film, the corneal epithelium presents a formidable barrier that protects the rest of the cornea from infection. Cells of the outer-most layer of the corneal epithelium have a "cornified envelope", i.e. a specialized layer of involucrin and loricrin proteins that are cross-linked to small proline-rich proteins (e.g. SPRRs 1-3), filaggrin, late-envelope proteins (e.g. LEP1), and peptidase inhibitor 3 (PI3 or elafin) within the cell and on the cell.[187-189] This creates a tough but flexible scaffold to strengthen the epithelial surface. The expression of SPRR genes increases after corneal stress (e.g. UV light exposure) and certain SPRRs also confer antioxidant protection since their cysteine residues are able to quench excessive reactive oxygen species (ROS) activity.[187, 190]

The multi-layered, stratified structure of the corneal epithelium is held together by tight junctions between cells (composed of the zonula occludens, occludin and the claudins) and hemidesmosome attachments to the basement membrane. Finally the epithelial basement membrane itself is able to provide a physical barrier to prevent micro-organisms from entering the corneal stroma; although the BM contains pores, they are smaller in diameter than most organisms and so physically block passage of pathogens.[191] In BK, *Pseudomonas*

aeruginosa in particular has evolved methods of traversing the corneal epithelium by either elaborating proteases to break down the basement membrane, or by directly invading and entering epithelial cells. [191, 192]

Pathogen recognition

Once inside the cornea, pathogenic organisms trigger an innate immune response through activation of pattern recognition receptors (PRRs).[174] In the normal cornea PRRs are present in the deepest epithelial cell layers (basal and wing cell layers), as well as in tissue-resident macrophages/dendritic cells in the anterior stroma, and keratocytes. Toll-like receptors (e.g TLR2 and 4)[193], c-type lectin receptors (e.g. Dectin-1[194-197]) or nod-like receptors (e.g. NLRP3[198]) have all been implicated in the detection of pathogens that cause human MK (see Table 4).

Increased gene expression of *TLR2*, *TLR4*, *TLR9*, *NLRP3* and *Dectin-1* (*CLEC7A*) as well as *NLRP3* was found in corneal scrape samples from patients with *Fusarium* and *Aspergillus* FK. Molecules present in the fungal hyphal cell wall (e.g. beta-glucans, chitins, alpha-mannans) are known to activate these PRRs. TLR2 is activated by several bacterial proteins, and TLR4 and TLR5 are activated by flagellin in *Pseudomonas aeruginosa*.[193, 199]

Activation of TLRs 2, 4, and Dectin-1 & -2, causes nuclear translocation of the transcription factor NFKB, and results increase in expression of genes that encode molecules involved in the innate immune response, e.g pro-inflammatory cytokines (e.g. *IL1B*, *TNF*), chemokines (e.g. *CXCL1*, *CXCL5*, *IL8*), and anti-microbial peptides (e.g. beta-defensin 2, *DEFB4*).[174, 194, 200, 201] IL1B, a "master regulator" of the inflammatory response, is produced as an inactive molecule which must be cleaved to be converted to its active form. Animal models and *in vitro* work have confirmed that in *Streptococcus pneumoniae* keratitis, the bacterial protein pneumolysin triggers activation of NLRP3 which then combines with ASC and caspase-1 (CASP1) to form the active inflammasome complex that is capable of cleaving pro-IL1B and pro-IL18.[198] IL1B is cleaved and activated predominantly by neutrophil elastases in *Pseudomonas aeruginosa* keratitis, rather than by the inflammasome NLRC4, which predominates in macrophages.[202] TLR5 activation by *Pseudomonas aeruginosa* also results in production of IL1B in human corneal fibroblasts.[199]

Leucocyte recruitment to the cornea

Chemokine production, e.g. CXCL1 and CXCL5 by activated corneal keratocytes[203] or IL8 by corneal epithelial cells[204], attracts neutrophils to the cornea within hours of onset of infection in MK. Neutrophils use cell adhesion molecules (ICAM1) to adhere to the keratocytes cellular processes and to migrate through the corneal stroma, as observed using HRT3 IVCM in live mouse models of corneal abrasion injury.[205, 206] Release of matrix metalloproteinases (MMPs) from neutrophils aids their migration through the stroma by breaking down adjacent collagens.[207, 208] Once at the site of infection, neutrophils begin to destroy the pathogen through mechanisms such as phagocytosis, release of antimicrobial peptides from granule proteins and use of reactive oxygen species.[209, 210] A specific subset of neutrophils that possess ROR-gamma-t are able to produce and respond to IL17, thus enhancing their reactive oxygen species production and ability to kill fungi in filamentous FK.[211] Neutrophil granule proteins with antimicrobial properties of importance in BK and FK include beta-defensins (e.g. beta defensin-2, also known as DEFB4)[212] and S100 proteins (e.g. S100A8 and S100A9)[213]. Neutrophils also employ the technique of NETosis to release a "net" of extracellular DNA embedded with neutrophil proteases over the pathogen (including bacteria, fungi and parasites) to attempt to destroy it.[214] This is a particularly important mechanism employed by neutrophils for large pathogens such as fungal hyphae that are too big to be successfully phagocytosed.[215] Although NET production can help to control the fungal load in some infections,[215] other pathogens are able to evade NETs, e.g. some keratitis-causing *Pseudomonas aeruginosa* strains.[216] Excessive NET production may also contribute to significant tissue damage in the host, through destruction of the extra-cellular matrix or by permeabilising adjacent host cells.[214, 215]

Adaptive immune responses

Although in late stage human FK, ulcerated corneal tissue is predominantly comprised of neutrophils and macrophages, there are still some CD3+ and CD4+ T-cells present (approximately 5% of infiltrating cells in corneal tissue from human *Fusarium* and *Aspergillus* FK), implying some element of a cell-mediated immune response.[217] Mouse models of *Aspergillus* and *Fusarium* keratitis have shown that Th17 cells arrive in the cornea first, before Th1 cells, probably due to earlier release of Th17 chemokines (CCL20, CCL22) by activated keratocytes before Th1 chemokines (CXCL9 and CXCL10) are released in the fungally-infected cornea.[218] In this model, Taylor *et al* found that by 24 hours post-infection, the source of most of the IL17A produced in the cornea was actually from neutrophils rather

than T-cells.[218] Although gene expression of Interferon-gamma (*IFNG*, a Th1 cytokine) was detected in these murine corneas with *Aspergillus* and *Fusarium* keratitis, and has also been detected in corneal scrapes from active human FK caused by the same fungi[217], blockade of IFNG (using anti-IFNG antibodies) did not have any significant impact on clinical severity of disease or fungal load in the animal model.[218] Th2 cells may not have much activity in later stage FK, since gene expression of *IL4*, a Th2-associated cytokine, was present at very low levels in corneal tissue from patients with *Aspergillus* and *Fusarium* keratitis obtained at the time of corneal transplantation surgery, in comparison with non-infected cadaveric control tissue.[217]

In contrast, in BK, T-cell responses may play a greater role in pathogen clearance and clinical severity than in FK. Mouse models of BK caused by *Pseudomonas aeruginosa* have shown that a Th1-dominated response (in C57BL/6 or B6 mice) ultimately results in corneal perforation within 3 days post-infection, whereas a Th2-dominated response (in BALB/c mice) results in lesser clinical severity and healing of the cornea.[219] Either removal of CD4+ T-cell activity (using monoclonal antibodies against this cell type) or blockade of IFNG both resulted in a delay in the onset of perforation in B6 mice until after 7 days post-infection.[219] Production of IFNG in murine *Pseudomonas* keratitis is important for adequate bacterial clearance and its production is driven by IL18 and IL12.[220, 221] IFNG is present in human corneal scrapes from BK due to *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*[222]. In addition to Th1 cells, natural killer cells are also a source of IFNG in *Pseudomonas* keratitis.[223]

Production of the anti-inflammatory cytokine IL10 in *Pseudomonas* keratitis may contribute to a dampening of macrophage and dendritic cell activity as well as Th1 responses, resulting in persistence of the pathogen and worse clinical severity.[224] Chronic PRR stimulation, either through prolonged presence of bacterial antigens (e.g. lipopolysaccharide acting upon NLRP3 in macrophages or TLR2/TLR4 in dendritic cells) or fungal antigens (e.g. fungal chitin acting upon TLR9) can also increase IL10 production, and thus reduce the host immune response to the pathogen.[225-227]

Wound healing versus tissue destruction in MK

Beyond pathogen clearance in microbial keratitis, specific healing phases occur in corneal epithelial cells and keratocytes with the aims of closing the wound and restoring corneal transparency. Epithelial cells become activated, and begin to produce a sequence of MMPs

over time that allow the cells from the wound margin to migrate as a sheet to close the epithelial defect.[228] These cells also increase their production of keratin type 6A (KRT6A), which may have intrinsic antibacterial properties as detected in mouse models of *Pseudomonas aeruginosa* keratitis.[229]

Keratocytes also become activated, and downregulate gene expression of normal keratocyte markers, such as proteoglycans (e.g. keratocan, *KERA*) and corneal crystallins (e.g. *ALDH1A1*, *ALDH3A1* and *TKT*), that help to biochemically maintain corneal transparency.[160, 230] Activated keratocytes, also known as corneal fibroblasts, can further differentiate into myofibroblasts upon exposure to growth factors such as TGFB2.[230, 231] Myofibroblasts are characterized by production of alpha smooth muscle actin, which allows them to effectively contract corneal wounds and promote wound healing.[232] Both activated keratocytes and myofibroblasts produce an initial scar tissue in the corneal stroma, mainly comprised of collagen types I and III, and begin to reform the corneal stroma.[231] However, disordered deposition of collagens in the stroma results in opaque scar tissue that can take many months to reorganize to improve translucency following an episode of MK.[233]

Finally, keratocytes can also undergo apoptosis as observed in human corneal tissue using TUNEL staining in BK, FK and AK.[163] This region of apoptosis extended beyond the border of inflammatory cells within the MK tissue, and often involved the posterior cornea.[163] Molecular mechanisms that trigger keratocyte apoptosis may be partly due to the pathogen, e.g. *Acanthamoeba*-induced direct cytopathic effect upon keratocytes[234], but may also be due to the host, e.g. IL1 or TNF released from injured host corneal epithelial cells inducing apoptosis in adjacent keratocytes.[235, 236]

Tissue destruction in MK

Much of the tissue damage in microbial keratitis may be caused by other neutrophil granule proteins such as the matrix metalloproteinases (MMPs).[237, 238] MMPs are also released by other cells in the cornea including corneal epithelial cells and activated keratocytes.[207] MMP9 in particular has a predilection for type IV collagen and so is able to break down basement membranes such as Descemet's membrane, thus predisposing toward corneal perforation.[207] Although MMP action is normally regulated by tissue inhibitors of MMPs (e.g. TIMPs 1-3), TIMP activity or gene expression has been shown to be much reduced in bacterial keratitis (caused by *Pseudomonas keratitis*) and filamentary fungal keratitis (caused by *Fusarium* sp. and

Aspergillus flavus) compared to the level of MMP activity or gene expression.[238-240] Anti-MMP therapeutic agents such as doxycycline or tetracycline have been used with some success to prevent corneal perforation in rabbit models of *Pseudomonas aeruginosa* keratitis, as well as in case reports of patients with BK caused by this pathogen.[241, 242]

Enzymes released by the pathogen itself may also damage the cornea. Serine proteinases are elaborated by clinical strains of *Aspergillus flavus* and *Fusarium solani*, many of which are able to break down collagens *in vitro*, and so may also be able to cause corneal collagen destruction in human FK.[98, 108] *Pseudomonas aeruginosa* can also produce proteases that can damage corneal tissue and worsen clinical severity of disease.[243]

1.15 Statement of the Problem

Several challenges currently exist in the diagnosis and management of MK. It is important to identify the causative organism early on so that the correct antimicrobial therapy can be started before excessive destruction of corneal tissue occurs. Culture, the current reference standard for making the microbiological diagnosis, can often take up to a week or longer for fungal and *Acanthamoeba* infections to be detected in the laboratory. Also, there remains a large number of patients for whom no organism can be detected ("culture negative" cases). IVCM allows immediate visualisation of fungi and *Acanthamoeba* cysts in the living cornea. The diagnostic accuracy of the high-resolution laser scanning IVCM has not as yet been ascertained. There are some reports to suggest that this imaging modality may also be used to monitor disease progression (at the cellular level),[154] but again this has not been formally examined in a larger series of MK patients.

1.16 Study Objectives

To formally evaluate *In Vivo* Confocal Microscopy for:

a. Distinguishing between fungal, bacterial and *Acanthamoeba* keratitis

b. Monitoring both the host and the organism response to anti-microbial treatment

1.17 Study Hypotheses

The major hypotheses for each of the studies in the results section of this PhD thesis are as follows:

Chapter 3:

1. There is a difference between bacterial, fungal and Acanthamoeba keratitis in terms of clinical features observed at slit lamp examination at presentation.

2. There is a difference between corneal ulcers that healed or improved versus those that perforated or worsened by day 21 post-enrolment in terms of socio-demographic risk factors or pathogenic organism detected on culture, light microscopy &/or IVCM.

Chapter 4:

1. The sensitivity and specificity of the HRT3 in vivo confocal microscope is equalrelative to that of light microscopy and/or culture of corneal ulcer scrapings is higher than that obtained with the Confoscan scanning slit in vivo confocal microscope (Chapter 4).

Chapter 5:

1. There is no difference in the branch angle observed in IVCM images from corneal ulcers that were culture-positive for *Fusarium* sp. versus those that were culture-positive for *Aspergillus* sp.

Chapter 6:

1. There is a difference between specific cellular or structural morphologies observed at presentation in IVCM images of ulcers that were culture-positive for bacterial keratitis, or culture-positive and/or IVCM-positive for fungal keratitis or *Acanthamoeba* keratitis.

Chapter 7:

1. There is a difference between specific cellular or structural morphologies observed at presentation versus final visit in IVCM images of culture-positive and/or IVCM-positive fungal keratitis that healed or improved versus those that perforated or worsened by day 21 post-enrolment.

1.18 Thesis Structure

This PhD thesis is written in the "research paper style". The results section consists of a series of 5 manuscripts each written in the format of a research paper; a cover-sheet precedes each manuscript and describes the role of the PhD candidate within each research study. Linking material written by Dr. Jaya Chidambaram has been included between each manuscript to aid in relating each research paper to the PhD objectives.

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1.20 Ethical Approvals

This study has been approved by the Aravind Eye Hospital Institutional Review Board, and the London School of Hygiene and Tropical Medicine Ethics Committee (Appendices 1a and 1b).

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Chapter 2

Methods

Digital photography of corneal ulcer in study participant

2.1 Study Design & Study Site

The research within this PhD was structured as a prospective cohort observational study.

Both studies were based in the Cornea Clinic at Aravind Eye Hospital which is situated in the city of Madurai in Tamil Nadu state in South India – the geographical location of Madurai is shown in Figure 1.

Figure 1: Map showing the location of Madurai in Tamil Nadu state, South India, as indicated by red marker (Map Data Source: Google).

Madurai City & District, Tamil Nadu State, India

Madurai city has a population of 1 million residents and is one of the largest cities in Tamil Nadu state.[1] It is the capital city of Madurai district (total population: 3 million residents).[1] Despite increasing urbanization in Madurai as well as many other districts in Tamil Nadu (see Figure 2), the districts surrounding Madurai remain predominantly rural (i.e. Sivagangai, Dindigul, Karur and Ramanathapuram).[1] The Census of India includes as its definition of an urban area that at least 75% of those of male gender who are within the working population are engaged in a non-agricultural occupation.[2] Agriculture forms one of the main sources of income for Tamil Nadu, taking into account all districts within the state.[3] As such many people in Tamil Nadu engaged in agricultural work are at risk of corneal trauma especially with vegetative matter that could trigger an episode of MK.

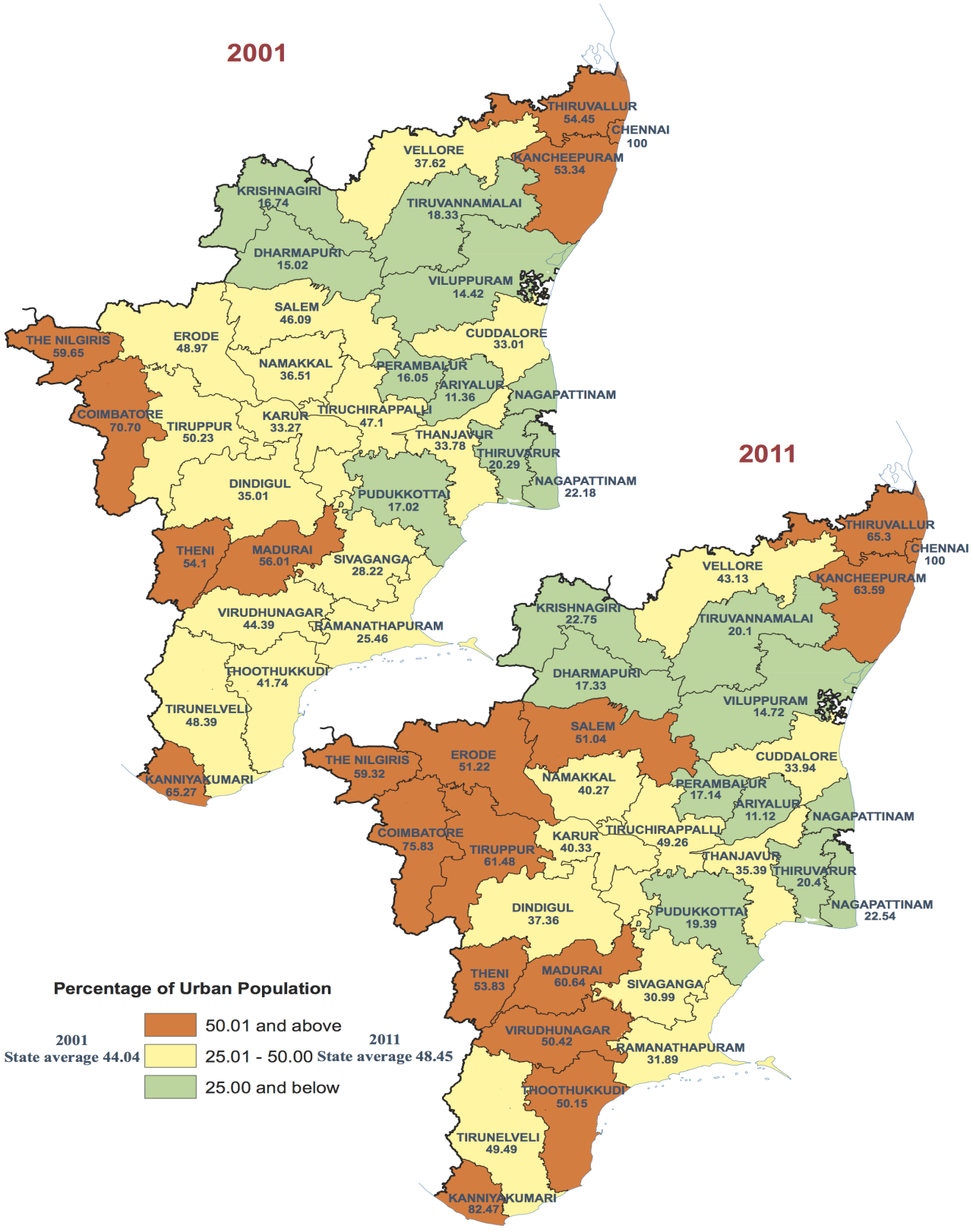


Figure 2: Map of percentage of urban versus rural population in districts of Tamil Nadu state (Source: S. Gopalakrishnan, Provisional population totals. Rural – urban distribution, Tamil Nadu, Census of India, 2011).

Aravind Eye Hospital, Madurai

Aravind Eye Hospital (AEH) was originally started in 1976 as an 11-bed hospital to provide eyecare for Madurai and its surrounding districts.[4] It has since expanded and now sees over 1.2 million outpatients per year[4] many of whom travel from other states in India to AEH for a specialist ophthalmic opinion; Figure 3 shows the AEH out-patient hospital in Madurai. Since opening AEH in Madurai, Aravind Eye Care System (AECS) recognised the burden of rural eye disease in the adjacent villages, and started outreach eye camps to diagnose treatable eye conditions such as microbial keratitis, and then to bring patients back to the main hospital in Madurai for treatment. In addition, AECS has since set up many village-based community eyecare centres as well as several eye hospitals in other parts of Tamil Nadu.

AEH, Madurai, has increasingly become a centre of excellence for research into MK, and FK in particular (Figure 4 shows the AEH Madurai Cornea Clinic). The cornea team at AEH has conducted several clinical trials in MK including the use of topical steroids in BK (in addition to antibiotic treatment) to reduce the scarring response and improve final visual outcomes,[5, 6] and the use of natamycin and voriconazole for the treatment of FK, both topically and in combination with oral treatment.[7, 8] AEH have also published on the both the human pathophysiology of BK and FK, as well as response of the bacterial and fungal pathogens.[9-12] The Cornea Department at AEH, Madurai, is a large tertiary referral centre that sees approximately 1700 corneal ulcer cases per year.[13] It is well served by an ocular microbiology department, fully functional eye bank (Aravind Rotary Eye Bank), dedicated in-patient beds for the cornea service and a corneal surgical operating theatre. For these reasons, we chose AEH as the base hospital for the MK studies in this PhD.

Figure 3: Aravind Eye Hospital, Madurai, Out-Patient Building (Source: Dr. NV Prajna, Director of Cornea Service, Aravind Eye Hospital, Madurai, India).

Figure 4: Cornea Department, Aravind Eye Hospital, Madurai, India. (Source: Dr. NV Prajna, Director of Cornea Service, Aravind Eye Hospital, Madurai, India)

2.2 Cohort Study Overview

Patients with clinically suspected MK of at least 3mm in diameter were enrolled from February 2012 to February 2013, as per the patient flow-chart in Figure 6. This allowed serial assessment of changes in the cornea, macroscopically using slit lamp biomicroscopy of clinical features, and microscopically using the in vivo confocal microscope. Informed consent was recorded in the study consent form (see Appendix 3). The section below on Ethical Considerations gives details of the informed consent process. A focused clinical history and slit lamp examination findings were recorded in a standardized data collection form (Appendix 4). Patient enrolment and patient flow for the Cohort study at the baseline visit and at follow-up visits are shown in Figures 5 and 6 respectively.

Figure 5: Patient Flow-chart for the Cohort Study Baseline Visit

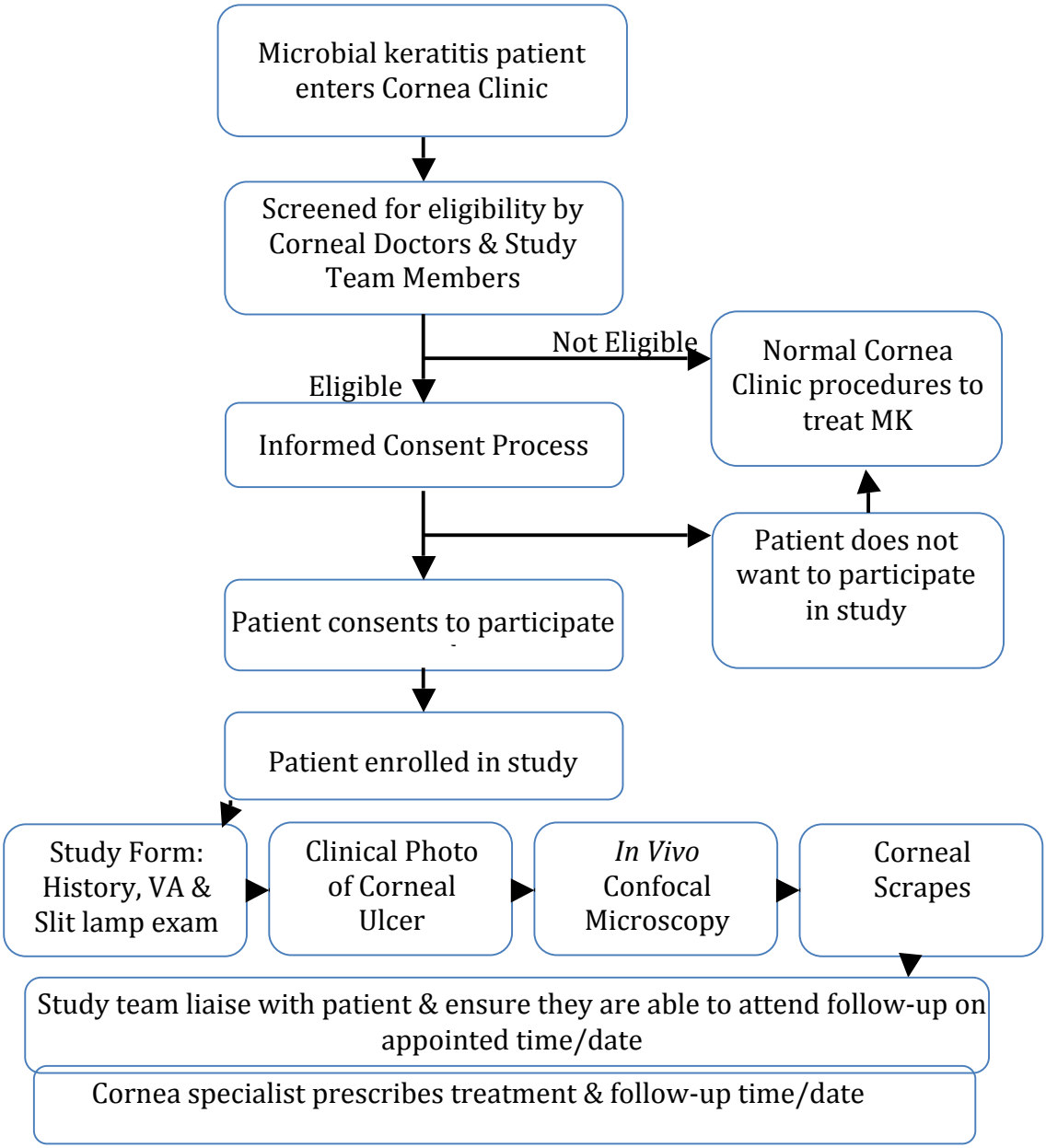
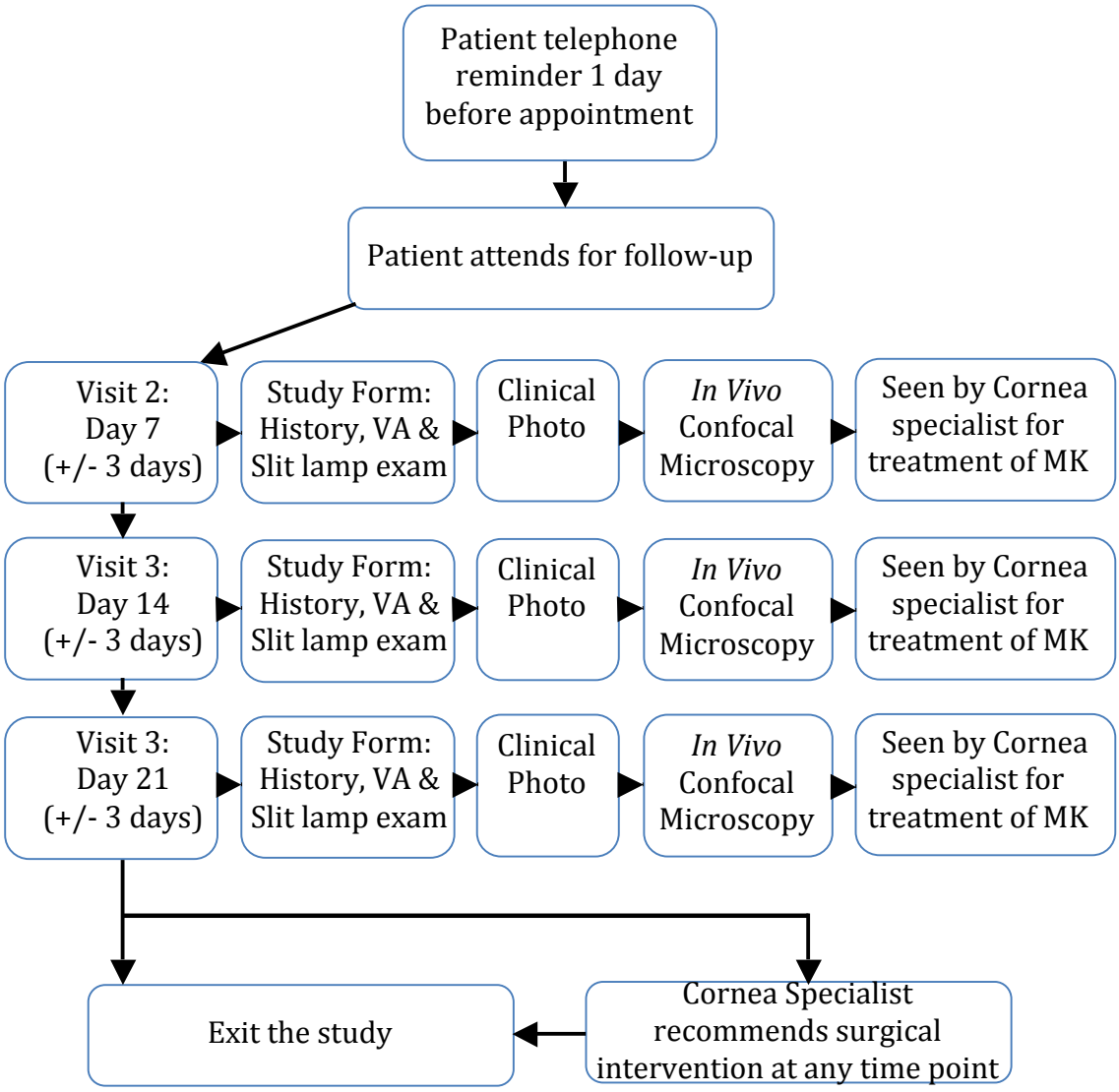


Figure 6: Patient Flow-chart for Follow-up Visits in the Cohort Study



Follow-Up Assessments

Patients recruited into the study were re-assessed with daily slit lamp examination by Aravind Cornea Fellows / Medical Officers / Cornea Consultants if they were admitted as in-patients in AEH. Scheduled follow-up visits for this study were arranged at days 7, 14 and 21 (see Figure 6). If the ulcer was particularly severe, an additional follow-up visit at either day 3 or 4 was scheduled for out-patients. At each visit, clinical assessment, clinical photography and IVCM was performed. Patients were invited to return at any time if they felt their symptoms were worsening, and these were recorded as unscheduled visits.

Slit Lamp Examination & Digital Photography of the Corneal Ulcer

At the initial visit and each follow-up visit, slit lamp biomicroscopy was performed and specific ulcer clinical signs were documented in the study form (as shown in Appendix 5). Ulcer epithelial defect size and stromal infiltrate diameter (longest diameter and its perpendicular) were measured using the slit lamp, as per standard methods used in other corneal ulcer clinical trials. Presence or absence of other features were also recorded, such as ulcer margin characteristics (well-defined or feathery), satellite lesions, surface pigment, endothelial plaques, anterior chamber activity or perforation. Ulcers were then photographed at each visit using a digital camera (Nikon D90) with standardized settings.

In vivo confocal microscopy (IVCM)

The HRT3 scanning laser ophthalmoscope with the Rostock Corneal Module (RCM) was used for IVCM imaging in this study (See Figure 7). A sterile, single-use disposable cap was used over the objective lens for each patient. Volume scans were performed which provide a sequential series of 400 x 400 μm images at consecutive 2 micron depths over a depth range of 80 μm.[14] The corneal ulcer was imaged at its centre, as well as its margins at the 12, 3, 6 and 9 o'clock positions. Volume scans were obtained starting from the superficial-most epithelial cells, then the Rostock corneal module was manually refocused and further overlapping volume scans obtained to image through the stroma to the endothelium where possible.

IVCM imaging was repeated at every follow-up visit, unless the cornea was deemed to be significantly thin (i.e. >80% thinning or a descemetocoele or corneal perforation observed in slit lamp examination), then IVCM was not performed. OCT imaging can be used to quantitatively assess corneal thickness in MK.[15] The majority of ulcers in this PhD study had a dense white infiltrate and many ulcers had involvement of the posterior cornea. OCT imaging in these cases produced an image with absence of the posterior half of the cornea due to most of the laser light being scattered by the optically dense white infiltrate, and so no light was detected by the OCT machine for the posterior aspect of the ulcer. As such it was not possible to use these abnormal images to calculate the corneal thickness. Hence, when we designed the study, we chose to estimate corneal thinning within the ulcer using slit lamp examination.

Figure 7: Patient positioning for imaging with the HRT3 in vivo confocal microscope with Rostock Corneal Module (RCM).

Corneal Scrapes and Microbiology

After IVCM imaging had been completed at the first visit, corneal scrapes were obtained from the base and leading edge of the corneal ulcer, as follows. Patients were asked to lie down on an examination couch and 0.5% proparacaine was applied to the cornea of the affected eye. A Kimura spatula was sterilized in a flame, allowed to immediately cool, then used to obtain material from the base and leading margin of the ulcer. The first scrapes were applied to two sterile glass slides (one for 10% potassium hydroxide staining to stain fungal filaments, the second for gram stain for bacteria). After this, further scrapes were directly applied to the surface of blood agar, chocolate agar and potato dextrose agar plates. If there was clinical suspicion of *Acanthamoeba* sp., a corneal scrape was also inoculated directly onto a non-nutrient agar plate that was overlaid with *E. Coli* immediately in the microbiology laboratory. Blood agar and chocolate agar plates were incubated at 37°C, aerobically, and monitored daily for two days for growth, then discarded if no growth had occurred; potato dextrose agar plates were incubated at 27°C. Fungal and *Acanthamoeba* cultures were assessed daily for growth and discarded at 7 days if no growth had occurred.[13] A plate was considered to have culture positivity if the same organism had grown on 2 or more of the solid media, or semi-confluent growth could be observed on one solid medium at the site of

inoculation along with the same organism identified on gram or giemsa-staining of the corneal scrape; culture positivity for *Staphylococcus epidermidis* or *diphtheroids* was determined only upon moderate growth of the organism on at least two solid media.[13]

Exclusion of Herpetic Keratitis

We excluded herpetic keratitis on the basis of clinical examination findings in slit lamp biomicroscopy and history of prior herpetic eye disease since we did not have access to a PCR-based lab test for herpes simplex virus types I or II, or herpes zoster virus. This methodology has been successfully used in previous BK and MK clinical trials based at Aravind Eye Hospital, Madurai, India.[5, 7] There is a theoretical possibility that patients presenting with mixed infection with bacterial or fungal keratitis as well as herpetic stromal keratitis without a prior history of herpetic eye disease, or any typical clinical features of herpetic epithelial or stromal keratitis may have had the possibility of being enrolled in this study, and may have theoretically contributed to the number of patients with poor outcome, (i.e. worsening clinically despite maximal antibiotic or antifungal therapy). However, the incidence of microbiologically-proven herpetic keratitis forms only approximately 7% of all acute microbial keratitis presenting to the Cornea Clinic in India, compared to up to 29% in the UK.[16, 17] As such, any cases of mixed infection with herpetic component, may have been minimal in number.

2.3 Specific Methods for IVCM Diagnostic Accuracy Study (Chapter 4)

At the end of the cohort study once all patients had been enrolled and all IVCM images had been collected, patient-identifying data and microbiological diagnoses were removed from each image, and patient images were allocated a random study identification number. The entire image-set for each patient were then shuffled into a random order prior to presentation to the IVCM grader. The IVCM grader looked through all randomized images obtained from the initial visit from all patients enrolled in the cohort study. All graders documented the definite presence, possible presence or absence of fungal filaments or acanthamoeba cysts in a Microsoft Access database that I created. More than 3 weeks after the initial grading, the images were shuffled again into another random order and 3 graders repeated the image grading. After the final grading session, data were exported into Stata for statistical analysis, which I performed under the supervision of my Statistician, Dr. Natasha Larke.

Sensitivity was calculated using the standard definition, i.e.

True positives (i.e. all those who were culture-positive AND IVCM-positive) ÷
[True positives + False negatives (i.e. all those who were IVCM-negative but culture-positive)]

Specificity was also calculated using the standard definition, i.e.

True negatives (i.e. all those who were culture-negative AND IVCM-negative) ÷
[True negatives + False positives (i.e. all those who were IVCM-positive but culture-negative)]

In this study, we prospectively included all patients with ulcer size ≥3mm who presented to the Cornea Clinic during the time period of the study. We did not create a fixed set of pre-selected IVCM images of "true positives", i.e. containing filamentous fungi, *Acanthamoeba* cysts, *Nocardia* filaments, or "true negatives" i.e. white blood cells within a corneal infiltrate or uninfected normal corneal epithelium, stroma or endothelium. This type of study, using pre-selected images, has already been performed by Hau *et al* in the past[18], and provides a sensitivity and specificity measurement for each individual grader which only applies for the fixed image-set, but does not test the ability of the grader to recognise fungi or acanthamoeba within the full range of images obtained from real-life corneal ulcers, which include ulcers at different stages of development. We designed a prospective cohort study to test the sensitivity and specificity of the graders when looking at the full range of real-life images obtained from microbial keratitis presenting to the cornea clinic over a 1 year period. This type of prospective cohort study has previously been used in ophthalmology to assess the diagnostic accuracy of new tests, for example see paper by Tufail *et al* who assessed the sensitivity and specificity of a new automated grading system in Diabetic Retinopathy (compared to human graders as the reference standard).[19] Overall, we conducted and reported our diagnostic accuracy study as per the Standards for Reporting of Diagnostic Accuracy Studies (STARD) guidelines.[20]

We did not include normal corneal control images, as we do not routinely image normal corneas in the Eye Clinic and we felt that inclusion of such images may confuse the graders resulting in abnormal sensitivity / specificity results. The IVCM images from our prospective cohort study included negative controls in the form of non-*Nocardia* bacterial keratitis. Also, although IVCM imaging of the fellow eye could be considered as a possibility to provide a control set of images, this does not prove to be the case in MK. Previous studies have found

significant changes in the fellow eye of patients with unilateral infective keratitis, i.e. reduction in sub-basal corneal nerve density and increase in dendritiform cell density in BK, FK and AK, as well as a reduction in endothelial cell count in herpetic keratitis.[21-23]

The results of the study by Hau *et al*, highlighted the spectrum of inter-grader variability, and the results of Vaddavalli *et al*, showed some but minimal intra-grader agreement after repeat grading of the same confoscan IVCM image-set several weeks later. Hence, we incorporated intra- and inter-grader agreement testing into our study design, and calculated kappa scores for each of these parameters.

2.4 Specific Methods for IVCM Branch Angle Study (Chapter 5)

For this study, I selected all of the IVCM images from ulcers that were culture-positive for either *Fusarium* sp. or *Aspergillus* sp., removed any patient-identifying data or microbiological diagnoses from the images, then applied a random identification number to identify the images from each patient. These were shuffled into a random order. In any image where I could visualise fungal filament branching, I exported the image from the Heidelberg Eye Explorer software into the ImageJ analysis software (National Institutes of Health, Bethesda, USA), and manually measured the branching angle using the Angle Measurement tool. Branch angle data was recorded in a Microsoft Excel spreadsheet and then statistical analysis was performed in Stata, under the supervision of my statisticians Dr. David Macleod and Dr. Natasha Larke.

2.5 Specific Methods for IVCM in BK, FK and AK (Chapter 6) and cellular morphology changes in FK (Chapter 7)

For Chapters 6 and 7, a single IVCM grader with 6 years of experience of grading IVCM images for corneal disease (i.e. myself) reviewed the randomised IVCM images from the baseline and final visits of all patients who had enrolled in the cohort study, with microbiological diagnoses and clinical outcome data removed. Images were graded according to presence or absence of specific IVCM cellular features as discussed in the Methods section in each manuscript in Chapters 6 & 7, with representative images for each morphological feature also given in these chapters. Statistical analysis for Chapter 7 included use of conditional logistic regression models to assess within each patient the change in morphological features (i.e. absent at baseline but then present in the final visit images). Logistic regression models were used to assess the morphological features associated with

BK, FK or AK in Chapter 6, and morphological features associated with clinical outcome in Chapter 7. In Chapters 6 and 7, multivariable regression analysis was used, i.e. multiple independent variables assessed for a single dependent variable.[24] Forwards stepwise regression was used in both chapters as explained in the Methods section in each individual results chapter, i.e. we set out with the null model then explanatory variables added and retained if strong statistical significance (i.e. $p<0.05$) was found using likelihood ratio testing compared to the first model (i.e with first variable retained).[25] Since the variance inflation factor (VIF) of variables included in the final models in these two chapters were all very close to 1.0, this implied no large effect of collinearity between variables, and therefore no need to remove any variables due to multi-collinearity effects.[26]

2.6 Other disease

If we detected any other eye disease that required treatment in any of our study participants, we referred the patient to the relevant ophthalmic specialist in Aravind Eye Hospital for assessment and further management. If other medical diseases were detected, we referred the study participant to the Consultant Physician at Aravind Eye Hospital in the first instance for appropriate investigation and treatment.

2.7 Ethical Considerations

Informed Consent

Informed consent involved a clear explanation of the study both in writing and verbally to the potential participant. The study consent form and patient information leaflets for Study A and Study B were available in English and Tamil language, and were read out to potential study participants in their language to enable them to understand the documents, even if they were unable to read (see Appendix 2 for patient information leaflets and Appendix 3 for consent forms). Consent was documented by either a signature or a thumbprint (in the case of illiterate patients) on the consent form as witnessed by a study team member. Patients were given every opportunity to ask any questions about the study to study team members. We ensured that patients understood that involvement into the study was entirely voluntary, and would not affect their standard corneal ulcer treatment in any way.

Potential risks to the individual

The eye examination performed in this study using a slit lamp was safe and part of routine clinical practice. Collection of corneal scrape samples was also part of standard care in the management of MK patients. We routinely used a drop of topical anaesthetic to the surface of the eye just before performing IVCM to prevent any discomfort. Corneal swab samples were collected very gently from the surface of the eye in a non-invasive manner. The IVCM examination and swab collections are associated with no or very minimal discomfort. All of these sample collection and IVCM methods have been used widely in ophthalmology before and are known to be very safe. Any patient with significant thinning of the cornea (>80%), or evidence of impending perforation (e.g. descemetocoele), or perforation did not undergo IVCM imaging.

2.8 Data Management

All of the study data forms and consent forms were kept securely locked in a lockable filing cabinet within the locked project office at AEH, Madurai. Data was entered into a computer at AEH, Madurai, which was password protected. This database was also password protected and was only accessible by the PI (Dr. Jaya Chidambaram) and the two study team members (Dr. Manisha Shah and Ms. Shanmugam Elakkiya).

2.9 Statistical Analysis

Methods for statistical analysis are described within the methods sections in each of the results chapters. Where multiple comparisons were used (i.e. Chapter 3), p-values were adjusted using Bonferroni correction.

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Chapter 3

Epidemiology, Risk Factors and Clinical Outcomes in Severe Microbial Keratitis in South India

Auto-rickshaw in front of In-patient Block, Aravind Eye Hospital, Madurai

3.1 Preamble

The manuscript in Chapter 3 introduces the cohort study population, and describes the epidemiological characteristics, clinical features and outcomes of the cohort study population. Further data from this study population is later used for the IVCM studies described in the remaining results chapters (i.e. Chapters 4-7).

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Student	Jaya Chidambaram
Principal Supervisor	Matthew Burton
Thesis Title	Studies in the Diagnosis & Pathophysiology of Severe Microbial Keratitis

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	Ophthalmic Epidemiology Journal		
When was the work published?	August 2018		
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SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I designed the study with assistance from co-authors Dr. M. Burton, Dr. M. Holland, Dr. Prajna, & Dr. Lalitha). I recruited study participants (with help from Ms. Elakkiya & Dr. Shah), performed ulcer slit lamp examinations and IVCM (along with co-authors Dr. P. Srikanthi & Dr. S. Lanjewar). I performed the statistical analysis. I wrote the manuscript, and included edits as suggested by co-authors.

Student Signature: [redacted] **Date: 8.8.18**
Supervisor Signature: **Date: 8.8.18**

Epidemiology, Risk Factors and Clinical Outcomes in Severe Microbial Keratitis in South India

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Running head: Epidemiology of severe microbial keratitis in India

Keywords: India, Epidemiology, *Acanthamoeba*, Corneal ulcer, Blindness, *Fusarium*, Microbial Keratitis, *Aspergillus*, *Streptococccus Pneumoniae*, Fungi.

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Conflicts of interest: None of the authors has a conflict of interest.

ABSTRACT

Purpose:

Here we report risk factors associated with outcome in severe bacterial (BK), fungal (FK) and *Acanthamoeba* keratitis (AK) in India.

Methods:

Prospective observational cohort study in Aravind Eye Hospital, India. Adults presenting with severe microbial keratitis (MK) were enrolled (size ≥3mm), and followed to 21 days post-enrolment. Ulcer clinical features were recorded at presentation. Outcomes by final visit were classified as good (completely healed or reduced infiltrate size) or poor (enlarged infiltrate size, perforated or surgery performed).

Results:

Of 252 participants with severe MK, 191 had FK, 18 AK, 19 BK, 4 mixed BK/FK and 20 were microbiologically-negative. Median age was 50 years (inter-quartile range, IQR,37-60 years), 64% were male, 63% agriculturalists, and 45% had no formal education. Corneal trauma occurred in 72% and median symptom duration before presentation was 7 days (IQR 5-15 days). Clinical features associated with FK were feathery margins ($p<0.001$), raised profile ($p=0.039$) or dry surface ($p=0.007$). Hypopyon was more likely in BK ($p=0.001$), ring infiltrate in AK ($p<0.001$). Ulcers with poor outcome (n=106/214) were more likely to be larger (OR 1.63, 95% CI: 1.30-2.05, $p<0.001$), involve the posterior cornea at presentation (OR 2.31, 95% CI 1.16-4.59, $p=0.017$), involve *Aspergillus* sp. (OR 3.23, 95% CI 1.26-8.25, $p=0.014$), or occur in females (OR 2.04, 95% CI 1.03-4.04, $p=0.04$). Even after treatment 34% (n=76/221) had severe visual impairment by the final visit.

Conclusions:

Severe MK occurred predominantly in agriculturalists post-corneal trauma and often had poor outcomes. Provision of community-based eyecare may allow earlier treatment and improve outcomes.

INTRODUCTION

Microbial keratitis (MK) can cause significant visual impairment, blindness or even loss of the eye, thus potentially having a major impact on the individual. In India, previous studies have highlighted that MK occurs more frequently in men, those in an agricultural occupation, and is more likely following trauma to the eye.[1,2] In particular, trauma with vegetative matter can predispose to fungal keratitis (FK), and exposure of the eyes to contaminated water is more often associated with *Acanthamoeba* keratitis (AK) in India. Although many studies in India have explored epidemiological risk factors for developing MK, very few have investigated risk factors for worse outcomes.

Lalitha et al studied patients with FK in South India and found that primary treatment failure (i.e. progressive worsening of the ulcer despite maximal medical treatment) or corneal perforation were associated with an infiltrate size >14 mm^2 or hypopyon at presentation, or if the ulcer was culture-positive for Aspergillus sp.[3] Rautaraya et al also studied bacterial keratits (BK) in East India and also found that larger ulcer size (>25mm^2) or poor visual acuity at presentation was associated with poor outcome, in addition to advancing age of the patient.[4] Titiyal et al specifically identified risk factors for corneal perforation in a cohort of predominantly bacterial corneal ulcers in North India; these authors found that a delay in the start of the antimicrobial treatment or in fact fortified antibiotics for BK was significantly associated with perforation.[5] One reason for delay in initiation of the correct treatment is the difficulty in making the diagnosis of BK, FK or AK. Clinical features may play a role in differentiating these causative organisms in keratitis. Prior studies have identified feathery margins, raised surface, satellite lesions and non-yellow infiltrate colour as more likely in FK[6,7], ring infiltrates in AK[8], and a well-defined border in BK (but with the exception of a wreath-like infiltrate in *Nocardia* keratitis), usually with a rapid-onset of ulceration in BK.[7,8]

However many patients present with large ulcers in India, and so the utility of these clinical signs in late-stage disease needs to be more fully evaluated.

In this study, we followed a cohort of patients presenting to Aravind Eye Hospital in South India with moderate-to-severe microbial keratitis in order to understand the clinical outcomes, and to assess epidemiological risk factors associated with these outcomes. We also studied the clinical features of bacterial, fungal and *Acanthamoeba* keratitis in order to determine if any of these features were specifically associated with a particular organism.

MATERIALS AND METHODS

Ethical approval was obtained for this study from the Indian Council of Medical Research, Aravind Eye Hospital Institutional Review Board and the London School of Hygiene and Tropical Medicine Ethics Committee. The tenets of the declaration of Helsinki were adhered to in conduct of this study. Study participants gave written informed consent; illiterate participants indicated their consent with a thumbprint on the consent form, witnessed by a member of the study team (approved by above ethics committees).

Between February 2012 and February 2013, patients aged $\geq$ 18 years presenting to the Cornea Clinic at Aravind Eye Hospital, Madurai, Tamil Nadu state, South India, with microbial keratitis (MK) were screened for eligibility to enter the study. Criteria for enrolment were presence of signs of MK at slit lamp examination (i.e. epithelial defect, underlying stromal infiltrate with signs of acute inflammation, e.g. conjunctival injection, anterior chamber cells, flare or hypopyon) with stromal infiltrate $\geq$3mm in longest diameter. Exclusion criteria were: evidence of herpetic keratitis (based on clinical signs on slit lamp biomicroscopy or past episodes); best-corrected Snellen visual acuity of 6/60 or worse in

fellow eye. At enrolment, patients were interviewed/examined and risk factor/clinical data were directly entered into a standard study form, which included socio-demographic details, focused clinical history and ulcer clinical features. A standardized digital photograph was taken of the ulcer at each clinic visit. Participants were reviewed again at day 7, 14 and 21 post-enrolment (+/- 3 days), or earlier if symptoms worsened. AK participants were followed-up in another research study.

At the baseline visit, participants underwent in vivo confocal microscopy (IVCM) using the HRT3 confocal microscope with Rostock Corneal Module, RCM, (Heidelberg Engineering, Germany) as previously described.[9] After IVCM, corneal scrapings were obtained from the base and leading edge of the ulcer for microbiological diagnosis, and processed/analyzed using standard methods described elsewhere.[9,10] Culture positivity was defined as a) growth of the same organism in at least 2 solid media, or b) semi-confluent growth in one solid medium at the inoculation site with the same organism identified on light microscopy.[10,11] Organisms grown in culture were speciated using standard methods.[11-13]. Bacterial keratitis was diagnosed only if there was a positive bacterial culture. Fungal keratitis was diagnosed if fungi were detected in light microscopy or in vivo confocal microscopy, and/or there was a positive fungal culture. *Acanthamoeba* keratitis was diagnosed if there was a positive culture and/or if cysts were seen within in vivo confocal microscopy or light microscopy images.

In Vivo Confocal Microscopy

Prior to scraping, the corneal ulcer was imaged with the HRT3 laser scanning confocal microscope with Rostock Corneal Module (Heidelberg Engineering, Germany) using methods previously described.[9,14] Briefly, the cornea was anesthetised with 0.5% proparacaine (Aurocaine, Aurolab, India), and the center and margins of the ulcer were

scanned from epithelium to endothelium where possible. All of the IVCM images obtained from all of the ulcers (i.e. including culture-negative ulcers and ulcers with positive bacterial culture results) were viewed by each grader independently and assessed for the presence/absence of *Acanthamoeba* cysts or fungal filaments. For any culture and light microscopy negative ulcers, if $\geq$3 out of 5 graders detected the definite presence of fungal filaments or *Acanthamoeba* cysts these ulcers were considered diagnostically positive for FK or AK respectively; graders were masked to the clinical and microbiological diagnosis.

Clinical Management and Outcomes

All patients were treated as per standard of care at Aravind Eye Hospital by the Cornea Consultant or Cornea Fellows. Primary treatment for fungal ulcers was intensive natamycin 5% eyedrops, for bacterial keratitis was moxifloxacin 0.5% eyedrops and for *Acanthamoeba* keratitis was 0.02% polyhexamethylene biguanide (PHMB). Appropriate oral antifungal or antibiotic was added if the ulcer was progressing close to the limbus. Patients with moderate-to-severe MK were admitted to the hospital for in-patient treatment at the initial visit, until eyedrop frequency had reduced enough that the patient was able to instill their eyedrop therapy at home. Medical treatment was changed at the discretion of the cornea specialist if the ulcer did not respond to primary treatment. Surgical intervention (i.e. corneal glue, intrastromal antifungal injection, therapeutic penetrating keratoplasty as required) was offered in the event of corneal perforation or clinical worsening despite maximal medical treatment. Clinical outcomes were defined as: "healed" if by the final visit there was <0.5mm epithelial defect and evidence of scar tissue either wholly or partly replacing the stromal infiltrate as observed with slit lamp biomicroscopy; "improving" if the stromal infiltrate diameter was less than 20% of the original diameter on slit lamp examination; "worse" if by the final visit the ulcer was the same size or larger than the

original diameter; "perforated" if there was evidence of full-thickness corneal perforation, or flat anterior chamber +/- evidence of suspected corneal perforation (i.e. iris plugging a possible corneal perforation site, or seidel-positive fluorescein dye test showing a leak of aqueous humor through suspected perforation). "Good" outcome was defined as a composite of those who had "healed" and "improved" outcomes. "Poor" outcome was a composite of those who had "worse" and "perforated" outcomes.

Statistical Analysis

Socio-demographic and clinical feature data were compared between organism groups and statistical significance assessed using the chi-square test for proportions and kruskall-wallis test for non-parametrically distributed variables (age, symptom duration, ulcer size), then bonferroni correction used to adjust p-values for multiple comparisons. Ulcer size was defined as the geometric mean of the longest diameter of the stromal infiltrate and its perpendicular diameter. Causative organism prevalence for severe MK over the study period was calculated using the denominator as the total number of eligible study participants with severe MK, and not the total number of patients with MK of any size seen at AEH over the study period. Multivariable logistic regression analysis was performed to assess clinical features associated with fungal keratitis, *Aspergillus* versus *Fusarium* keratitis, as well as risk factors associated with worse versus good outcome (adjusted for age and gender). Forwards stepwise regression analysis was used, and variables assessed for significance against the full model using likelihood ratio testing. A significance level of $p<0.1$ was used for initial inclusion in the model and for $p\leq0.05$ in likelihood ratio testing (against the full model) for retention in the model. All analyses were performed in Stata v12.1 (StataCorp, Texas, USA).

RESULTS

Among the 252 eligible participants enrolled in the study, the organisms detected were fungi in 191 (76%), *Acanthamoeba* sp. in 18 (7%), bacteria in 19 (7%), microbiologically-negative (i.e. no organism detected by culture, light microscopy or IVCM) in 20 (8%), and mixed bacterial/fungal infection in 4 (2%). In the pure fungal cultures, the most frequently detected fungi were *Fusarium* sp. (n=75/191; 39%) and *Aspergillus* sp. (n=35/191; 18%). In pure bacterial ulcers, the main bacteria identified were *Streptococcus pneumoniae* (n=9/19; 47%), *Pseudomonas aeruginosa* (n=4/19; 21%), and *Nocardia* sp. (n=3/19; 16%).

Socio-demographic risk factors for Bacterial, Fungal and Acanthamoeba Keratitis

The median age of all participants was 50 years (inter-quartile range 37-60 years), of whom 64% were male (n=162). Most patients were agricultural workers (63%, n=159/251), and 45% of participants had not undergone any formal education (n=111/246). There were no significant differences in age, gender, agricultural occupation, or educational status between the FK, BK, AK and microbiologically-negative groups (Table 1). A greater proportion of the microbiologically-negative group were current tobacco users compared to BK, FK and AK (33%, n=6/18; p=0.047 - see Table 1). Corneal trauma was sustained in 72% of participants immediately prior to onset of the ulcer (n=181/252). The source of trauma was vegetative matter in 46% (n=82/180); vegetative trauma occurred more frequently in the BK group (76% of BK patients with corneal trauma, n=10/13), rather than FK, AK or microbiologically-negative ulcers (p=0.033, Table1) and was mainly in ulcers caused by *Streptococcus pneumoniae* (n=5/10) or *Nocardia* sp. (n=2/10).

Table 1: Socio-demographic and clinical risk factors associated with bacterial, fungal & *Acanthamoeba* keratitis at presentation

Risk Factor	All Microbial keratitis (n=252)	Bacterial keratitis (n=19)	Fungal keratitis (n=191)	*Acanthamoeba* keratitis (n=18)	No organism (n=20)	p-value*
Socio-demographic						
Age, years (median, IQR)	50 (37-60)	60 (35-65)	50 (36-58)	39 (34-55)	53 (43-61)	0.066
Gender (% male)	162 (64%)	12 (63%)	123 (64%)	11 (61%)	13 (65%)	0.993
Occupation: no. agricultural workers	159/251 (63%)	15 (79%)	121 (64%)	11 (61%)	10 (50%)	0.311
Had primary education or greater	135/246 (55%)	8 (42%)	107/186 (57%)	9/17 (53%)	10 (50%)	0.577
Current tobacco use	34/234 (14%)	4/16 (25%)	23/178 (13%)	1 (6%)	6/18 (33%)	0.047
History						
Symptom duration before presentation to AEH, days (median, range)	7 (5-15)	9 (5-15)	7 (4-11)	30 (20-60)	10 (7-21)	<0.001
Symptom duration before presentation to any healthcare provider (HCP), days (median, range)	2 (0-3)	2 (0.5-5)	2 (0-3)	2 (0-7)	0 (0-3)	0.547
History of eye trauma	181 (72%)	13 (68%)	134 (70%)	13 (72%)	17 (85%)	0.563
Trauma with vegetative matter	82/251 (33%)	10/13 (77%)	56/133 (42%)	3/13 (23%)	9/17 (53%)	0.033
Prior antifungal use	125/220 (57%)	7/16 (44%)	98/169 (58%)	10 (59%)	8/15 (53%)	0.727
Prior antibiotic use	162/220 (74%)	11/16 (69%)	123/169 (73%)	14/17 (82%)	11/15 (71%)	0.825
Prior steroid use	25/220 (11%)	1/16 (6%)	16/169 (9%)	7/17 (41%)	1/15 (7%)	0.001
Traditional medicine use	48 (19%)	4 (21%)	37 (19%)	5 (28%)	2 (10%)	0.578
Plant-based traditional medicine used	21/47 (45%)	2/4 (50%)	15/36 (42%)	3/5 (60%)	1/2 (50%)	0.879
Prior MK in affected eye	9 (4%)	1 (5%)	8 (4%)	0 (0%)	0 (0%)	0.627
Diabetes mellitus	17 (7%)	0 (0%)	15 (8%)	0 (0%)	1 (5%)	0.356
Initial Clinical Features						
Stromal infiltrate size, mm^2 (median, IQR)	4.5 (3.5-5.9)	3.9 (3.2-6.1)	4.5 (3.3-5.5)	6.8 (5.3-8.0)	5.2 (4.0-6.1)	<0.001
Posterior 1/3 of cornea affected at presentation	168/251 (67%)	14 (74%)	123 (64%)	12 (71%)	17 (85%)	0.261
Entropion, Ectropion or Lagophthalmos present	12 (5%)	2 (10%)	8 (4%)	0 (0%)	2 (10%)	0.310
Blocked Tear Duct	19 (7%)	4 (21%)	12 (6%)	0 (0%)	3 (15%)	0.039

*Bacterial, fungal & *Acanthamoeba* groups compared for statistically significant differences using chi-square test (for proportions) or kruskall-wallis test (non-parametrically distributed variables). P-value <0.0025 considered statistically significant as per Bonferroni correction (n=20 tests).

In all ulcers, the median symptom duration prior to presentation was 7 days (interquartile range, IQR: 5-15 days), but this was significantly longer in AK compared to all other groups (30 days, IQR 20-60 days, p<0.001; Table 1), as we have previously reported.[9]

The majority of patients (90%, n=226/252) had sought help from another healthcare provider (HCP) prior to presentation at AEH, including other ophthalmologists (65%, n=163/252), general physicians (8%, n=21/252), pharmacists (7%, n=17/252) and traditional medicine healers (19%, n=48/252); 25 patients (10%) saw more than one HCP. At presentation, 220 patients were using antimicrobial treatment: 71 were using topical antibiotics, 34 topical antifungals alone and 91 were using both antifungal and antibiotic. Topical steroids were being used by twenty-five patients at presentation, and this was particularly the case in AK (n=7/18; 39%, p<0.001; Table 1). Traditional medicines were used as initial treatment for the ulcer in 19% of participants (n=48/252); the most frequent remedies used were topically applied breast milk (n=24) or plant-based oils/flower extracts (n=22, e.g. castor oil, n=8). Use of plant-based traditional medicines was not significantly associated with FK or any other causative organism (p=0.879, Table 1).

Patients reported their main reasons for delay in seeing an ophthalmologist as a lack of pain or reduced vision causing the patient to believe the eye problem was not serious (51%, n=69/134), lack of availability of a person to escort the patient to the eye hospital (32%, n=43/134), lack of finances for travel costs (6%, n=8/134), other family, work or travel commitments (7%, n=9/134), and lack of knowledge of local eyecare services (4%, n=5/134).

Ocular and systemic risk factors

With regards to systemic medical conditions, diabetes mellitus was present in 17 patients (Table 1), 15 of whom had FK. Presence of a blocked tear duct occurred more frequently in BK than in FK, AK or microbiologically-negative ulcers as shown in Table 1 (21% in BK, n=4/19, p=0.039). All of these bacterial ulcers were culture-positive for *Streptococcus pneumoniae*.

Overall, presenting best-corrected visual acuity in the affected eye fulfilled the WHO criteria for moderate or severe visual impairment in 71.3% of patients (n=174/244; detailed in Table 2).

Table 2: Proportion of study participants with mild, moderate or severe visual impairment (VI), or blindness in affected eye at presentation and at final visit, as per World Health Organisation criteria.

Risk Factor	Totals	Bacterial Keratitis (BK)	Fungal Keratitis (FK)	*Acanth-amoeba* Keratitis (AK)	No organism	p-value*
Visual Acuity (snellen VA) at Present-ation	**N=244**	**N=19**	**N=189**	**N=16**	**N=20**	
Normal (<6/9)	22 (9%)	1 (5%)	19 (10%)	1 (6%)	1 (5%)	0.773
Mild VI (6/9 to 6/18)	24 (10%)	1 (5%)	21 (11%)	0 (0%)	2 (10%)	0.468
Moderate VI (>6/18 to 6/60)	35 (14%)	2 (10%)	31 (16%)	1 (6%)	1 (5%)	0.369
Severe VI (>6/60 to 3/60)	139 (57%)	13 (68%)	102 (54%)	11 (69%)	13 (65%)	0.370
Blind (>3/60)	24 (10%)	2 (10%)	16 (8%)	3 (19%)	3 (15%)	0.485
VA at Final Visit	**N=221**	**N=18**	**N=185**	**-**	**N=18**	
Normal	32 (14%)	0 (0%)	30 (16%)		2 (11%)	0.155
Mild VI	43 (19%)	3 (17%)	36 (19%)	-	4 (22%)	0.915
Moderate VI	34 (15%)	2 (11%)	28 (15%)	-	4 (22%)	0.641
Severe VI	76 (34%)	7 (39%)	65 (35%)	-	4 (22%)	0.489
Blind	36 (16%)	6 (33%)	26 (14%)	-	4 (22%)	0.413

*Proportion of BK, FK, AK groups within each visual acuity category compared with all others for statistically significant differences using chi-square test.

In the unaffected eye at presentation, 10.7% of patients had moderate visual impairment (n=26/244). Stromal infiltrate size was also large at presentation for all MK patients (median 4.5mm^2, IQR, 3.5-5.9mm^2) and with deep involvement of the posterior third of the cornea in 67% of ulcers (n=168/251). Ulcer size was significantly greater in AK than in BK, FK or microbiologically-negative ulcers (median 6.8 mm^2 stromal infiltrate diameter, IQR 5.3-8.0 mm^2, p<0.001, Table 1).

Ulcer Appearance at Presentation and Causative Organism

At the first visit, fungal ulcers were more likely to have feathery margins (p<0.001), raised profile (p=0.039) and dry texture of surface slough (p=0.007) compared to the BK and AK groups in univariable analysis (Table 3). Logistic regression analysis of fungal ulcers versus all others showed that feathery margins were strongly associated with fungal ulcers (OR 4.47, 95% CI 2.10-9.50, p<0.001) and ring infiltrate associated with non-fungal ulcers (OR 0.43, 95% CI 0.20-0.92, p=0.029). A higher proportion of AK patients had a ring infiltrate (89%, n=16/18), than in FK or BK (p<0.001). Bacterial ulcers were more likely to have a hypopyon (p=0.002, Table 3), and in fact all ulcers caused by *Streptococcus pneumoniae* in this study had a hypopyon at presentation (n=10 [9 pure BK, 1 mixed BK/FK]).

Within FK, comparing ulcers caused by *Fusarium* sp. with *Aspergillus* sp. we found that *Fusarium* ulcers were more likely to have feathery margins (OR 4.55, 95% CI 1.92-10.75, p=0.001) or a non-yellow infiltrate (OR 4.42, 95% CI 1.78-10.95, p=0.001) in the univariable logistic regression analysis. *Aspergillus* ulcers were more likely to have a raised surface (OR 2.67, 95% CI 1.10-6.44, p=0.029) or a hypopyon (OR 2.98, 95% CI 1.16-7.67, p=0.024) compared to *Fusarium* ulcers in univariable analysis.

Table 3: Clinical features in bacterial, fungal and *Acanthamoeba* keratitis at presentation.

Ulcer Appearance	Bacterial Keratitis, BK (n=19)	Fungal Keratitis, FK (n=191)	*Acanthamoeba* Keratitis, AK (n=18)	p-value*
Feathery margin	5 (26%)	134 (70%)	2 (11%)	<0.001
Raised profile	3 (16%)	48 (25%)	0 (0%)	0.039
Dry texture of slough	1 (5%)	77 (40%)	5 (28%)	0.007
Satellite lesions	5 (26%)	70 (37%)	2 (11%)	0.070
Non-yellow infiltrate	12 (63%)	141 (74%)	16 (89%)	0.197
Pigmented ulcer	0 (0%)	9 (5%)	0 (0%)	0.404
Ring infiltrate	8 (42%)	54 (28%)	16 (89%)	<0.001
Perineural infiltrate	0 (0%)	1 (0.5%)	1 (6%)	0.083
Corneal neovascularisation	5 (26%)	22 (11%)	3 (17%)	0.172
DM Folds	6 (32%)	94 (49%)	6 (33%)	0.172
Endothelial plaque	10 (53%)	75 (39%)	4 (22%)	0.164
Hyopopyon	17 (89%)	120 (63%)	6 (33%)	0.002
Cells and/or flare in AC	11/12 (92%)	102/148 (69%)	6/12 (50%)	0.085
Keratic precipitates	6 (32%)	55/183 (30%)	4/16 (25%)	0.900

*Comparison of all 3 groups (BK, FK, AK) to assess for statistically significant differences using chi square test.

There was no significant difference between *Fusarium* or *Aspergillus* ulcers for the presence of satellite lesions, ring infiltrate, Descemet's membrane folds or an endothelial plaque. Multivariable analysis found that only feathery margins (OR 3.56, 95% CI 1.45-8.76, p=0.006) and non-yellow infiltrate colour (OR 3.28, 95% CI 1.26-8.56, p=0.015) remained significantly associated with *Fusarium* infection.

Risk factors associated with clinical outcome

Clinical outcome data was obtained for 227 participants; no outcome data was available for 7 participants who were lost to follow-up after the first visit (n=5 FK, n=1 BK, n=1 microbiologically-negative) or for the 18 participants with AK (who were followed in another study). By the final visit, 110 participants had a good outcome (40 healed and 70

were improving) and 117 had a poor outcome (i.e. perforated/required corneal glue, n=42, or worsened, n=75). Overall, by the final visit, best-corrected visual acuity measurements found that most MK patients had severe visual impairment (severe VI, 34%, n=76/221), and 16% (n=36/221) fulfilled the WHO criteria for blindness (Table 2).

Participants with a poor outcome presented with a significantly longer symptom duration (median 9 days vs 7 days in good outcome group, p<0.001; Table 4), worse visual acuity (median logMAR 1.8 vs 1.7 in good outcome, p<0.001; Table 4) and larger ulcers (median infiltrate size 5.2mm2 vs 3.8mm2, p<0.001; Table 4). A greater proportion of those with a poor outcome had involvement of the posterior cornea at presentation (80% vs 53%, p<0.001; Table 4) or presence of a hypopyon (74% vs 55% of those with a good outcome, p=0.004).

Multivariable analysis revealed the main epidemiological risk factors most significantly associated with poor outcome were female gender (OR 2.04, 95% CI: 1.03-4.04, p=0.04; Table 5), no formal education (OR 2.30, 95% CI: 1.14-4.62, p=0.019; Table 5) and symptom duration (OR 1.05, 95% CI: 1.00-1.10, p=0.032; Table 5). Ulcer features also associated with poor outcome were larger ulcer size at presentation (OR 1.63, 95% CI: 1.30-2.05, p<0.001), as well as involvement of the posterior cornea (OR 2.31, 95% CI 1.16-4.59, p=0.017) and culture positive result for *Aspergillus* sp. (OR 3.23, 95% CI 1.26-8.25, p=0.014; Table 5). Several socio-demographic features were significantly associated with good outcome, including younger age at presentation (median age 45 years vs 53 years in poor outcome group, p=0.036, Table 4), male gender (71% versus 58% with poor outcome, p=0.031), and educational status of primary school level or beyond (65% vs 46% in poor outcome, p=0.004).

Table 4: Risk factors associated with clinical outcomes in severe microbial keratitis (*groups compared with chi-square test for proportions and kruskall-wallis for continuous non-parametrically distributed variables; p-value <0.002 considered statistically significant as per Bonferroni correction, n=25 tests).

Outcome Risk Factor	Worse (n=117)	Good (n=110)	p-value*
Socio-demographic			
Age, years (median, IQR)	53 (40-60)	45 (35-57)	0.036
Gender (no. male, %)	68 (58%)	79 (71%)	0.031
Occupation: agricultural	82 (70%)	62/109 (57%)	0.039
Primary education or more	53 (46%)	69 (65%)	0.004
Current smoker/tobacco use	14 (13%)	18 (17%)	0.392
History			
Symptom duration, days (median, range)	9 (5-15)	7 (4-10)	<0.001
Any eye trauma	84 (72%)	80 (73%)	0.875
Trauma with vegetative matter	44 (38%)	32/109 (29%)	0.190
Prior antifungal use	61/102 (60%)	50/94 (53%)	0.351
Prior antibiotic use	77 (75%)	64 (68%)	0.249
Prior steroid use	9 (9%)	8 (8%)	0.938
Traditional medicine use	20 (17%)	22 (20%)	0.573
Plant-based	7/19 (37%)	10/22 (45%)	0.577
Seen by another healthcare provider first	103 (88%)	98 (89%)	0.803
Prior microbial keratitis	6 (5%)	3 (3%)	0.354
Diabetes mellitus	12 (10%)	4 (4%)	0.051
Clinical Features at Presentation			
Best-corrected visual acuity affected eye (median logMAR, IQR)	1.8 (1.7-1.8)	1.7 (0.46-1.8)	<0.001
Stromal infiltrate size, mm^2 (median, IQR)	5.2 (4.0-6.5)	3.8 (3.1-4.7)	<0.001
Posterior 1/3 of cornea involved (N, %)	94 (80%)	58 (53%)	<0.001
Presence of hypopyon	86 (74%)	61 (55%)	0.004
Organism			
Bacteria (excluding mixed BK/FK)	10 (9%)	8 (7%)	0.695
Streptococcus pneumoniae	7 (7%)	2 (2%)	0.108
Fungi (excluding mixed BK/FK)	95 (86%)	91 (84%)	0.661
Fusarium spp.	28 (24%)	46 (42%)	0.004
Aspergillus spp.	22 (19%)	8 (7%)	0.010

Table 5: Univariate and multivariate logistic regression analysis of risk factors associated with worse outcome in severe microbial keratitis (*adjusted for age and gender).

Risk Factor	Univariable OR* (95% CI)	p-value	Multivariable OR (95% CI)	p-value
Age	1.09 (1.00-1.04)	0.048	0.99 (0.97-1.01)	0.539
Female gender	1.84 (1.05-3.20)	0.032	2.04 (1.03-4.04)	0.040
Presenting ulcer infiltrate size (mm^2)	1.80 (1.45-2.23)	<0.001	1.63 (1.30-2.05)	<0.001
Aspergillus sp. isolated	2.87 (1.19-6.88)	0.018	3.23 (1.26-8.25)	0.014
Posterior corneal involvement	3.33 (1.82-6.11)	<0.001	2.31 (1.16-4.59)	0.017
No formal education	1.68 (0.92-3.05)	0.089	2.30 (1.14-4.62)	0.019
Symptom duration (days)	1.07 (1.03-1.12)	0.001	1.05 (1.00-1.10)	0.032
Vegetative trauma	1.68 (0.94-3.02)	0.079	-	-

Analysis of the outcomes for the most frequent causative organisms showed that 62% of *Fusarium* sp. ulcers had a good outcome (n=46/74), versus only 23% in *Aspergillus* sp. ulcers (n=8/35), and 22% in *Streptococcus pneumoniae* ulcers (n=2/9, i.e. n=2/8 pure *Streptococcus pneumoniae* keratitis and n=0/1 mixed infection with fungus; Table 6). With regards to perforation, 18% of *Fusarium* ulcers perforated (n=13/74), versus 20% of *Aspergillus* ulcers (n=7/35) and 56% of *Streptococcus pneumoniae* ulcers perforated or required corneal glue (n=5/9, i.e. n=4/8 pure *Streptococcus pneumoniae* keratitis and n=1/1 mixed infection with fungus; Table 6). Overall, patients who developed corneal perforation had significantly longer symptom duration prior to presentation (median 10 days, IQR 6-15, p=0.008) compared to all other ulcers (median 7 days, IQR 4-10). The ulcer itself was significantly larger at presentation in the perforated group (5.3mm^2 in perforated ulcers, IQR 3.9-6.9mm^2 vs 4.2mm^2 all others, IQR 3.3-5.3mm^2, p=0.001) and a greater proportion of perforated ulcers already involved the posterior third of the cornea at the first visit (90% in perforated ulcers vs 62% all others, p<0.001). Initial visual acuity was also significantly poorer in the perforation group (median logMAR 1.8, IQR 1.7-1.8, p=0.001).

Table 6: Organisms identified by culture, light microscopy and *in vivo* confocal microscopy by clinical outcome.

Organisms	Perforated or Glued (n=42)	Worse (n=75)	Improving (n=70)	Healed (n=40)
Bacteria (n=18)	**n=6**	**n=4**	**n=4**	**n=4**
Streptococcus pneumoniae	4	2	1	1
Streptococcus viridans	1	0	0	0
Staphylococcus epidermidis	0	1	0	0
Nocardia sp.	0	0	2	1
Pseudomonas aeruginosa	1	1	0	2
Aeromonas sp.	0	0	1	0
Fungi (n=186)	**n=32**	**n=63**	**n=59**	**n=32**
Aspergillus sp.	7	20	7	1
Fusarium sp.	13	15	25	21
Alternaria sp.	0	0	1	0
Bipolaris sp.	0	0	1	0
Curvularia sp.	0	1	2	1
Cylindrocarpon sp.	1	1	0	0
Exserohilum sp.	2	2	0	1
Lasiodiplodia sp.	0	1	1	0
Unidentified hyaline fungi	1	10	4	0
Unidentified dematiaceous fungi	2	3	5	1
Light microscopy +ve for fungi, culture negative	5	7	10	5
Light microscopy & culture negative for fungi, but IVCM +ve for fungi	1	3	3	2
Mixed bacterial/fungal* (n=4)	**n=1**	**n=2**	**n=1**	**n=0**
Streptococcus pneumoniae	1	0	0	0
Streptococcus viridans	0	1	1	0
Staphylococcus epidermidis	0	1	0	0
Micro/IVCM Negative (n=19)	**n=3**	**n=6**	**n=6**	**n=4**
Light microscopy, culture & IVCM negative for any organism	3	6	6	4

*Mixed infections were culture-positive for bacteria (species shown in table) and also positive for fungi detected only in light microscopy and/or *in vivo* confocal microscopy.

DISCUSSION

In this prospective study, we have explored epidemiological risk factors and clinical features associated with severe BK, FK and AK, and described MK clinical outcomes. In severe MK, the outcomes for many patients can be poor, with corneal perforation in up to 30% and loss of the eye in up to 25%.[15,16] Numerous prior publications have examined the epidemiology of MK throughout India. Our results also show that agricultural workers are predominantly affected and that exposure to corneal trauma, frequently with vegetative matter, is an important risk factor for onset of MK. These individuals were often male, and within the working age-group in this study. Most previous reports have noted an association between vegetative trauma and eyes with fungal infection, however our findings indicate an association with bacterial keratitis.[2,10] This highlights the fact that corneal injury with vegetative matter may provide a route of entry for other pathogens such as *Streptococcus pneumoniae* that are not classically known as plant pathogens. Although most participants in this study presented to the eye hospital approximately 7 days or more after onset of symptoms, many had visited a HCP much earlier, sometimes within the first day of developing symptoms. However, many patients were not placed on appropriate antimicrobial treatment following this initial assessment; particularly in the case of AK patients, many were started on topical steroids without anti-acanthamoeba treatment prior to presentation at the eye hospital, which has been associated with worse visual outcome in AK.[17]

We found in our study population that poor outcomes occurred predominantly in those of an older age, female gender and with no formal education. Tamil Nadu has relatively high literacy rate compared to other states in India (80.3%, ranked 14 out of all 35 states in the 2011 Census of India data).[18] However, within the rural districts of Tamil Nadu, there

remains a lower level of literacy with gender gap, e.g. Erode where overall literacy rate was 65%, but only 56% of women were literate as per the 2011 Census data.[18] Local eyecare service providers could aim to reduce these health inequalities in the future when planning services. In addition, larger ulcers with longer symptom duration had a poor outcome, as reported by others[3,4], highlighting the need for patient education to encourage individuals to seek eyecare early on in the course of disease to improve final visual outcomes. Many patients reported that they either did not take their initial ocular symptoms seriously, or that they had difficulties in organizing travel to the eye hospital along with a person to accompany them. Other studies have analyzed barriers in the uptake of eyecare services in rural India, and found similar reasons to those reported in our study.[19,20] Provision of antimicrobial eyedrop therapy such as natamycin in community-based clinics may alleviate these issues, and allow correct antimicrobial treatment to be instituted under ophthalmic supervision early on in the course of disease.

We have found specific epidemiological risk factors (female gender, no formal education) and ulcer-related risk factors (culture-positive for *Aspergillus* sp., involvement of the posterior cornea, larger ulcer size at presentation) in the multivariable analysis that were associated with poor outcome in our South Indian, mainly rural study population with moderate-to-large microbial keratitis. The greatest risk factor for poor outcome in our study was *Aspergillus* keratitis. Presence of one or more of these specific risk factors at presentation may be of prognostic value. Prognostic risk scoring models have been developed and used successfully for ocular trauma as well as other diseases, e.g. myocardial infarction or stroke, and can help the clinician to identify more highly at-risk patients at presentation.[21,22] Future studies with larger samples size could be used to formally assess the risk factors that we have described in the form of a prognostic risk score for MK.

The etiological agent in larger ulcers can be difficult to diagnose based on clinical features alone.[7] We found that even in moderate-to-large ulcers, the presence of feathery margins, raised profile and dry surface are more likely to occur in FK, as also shown by others.[6,7] Unlike previous studies that have assessed features specific to *Fusarium* sp. or *Aspergillus* sp., we found that feathery margins were more associated with *Fusarium* sp. and raised profile or hypopyon with *Aspergillus* sp.[23] Most of the bacterial ulcers in this study were caused by *Streptococcus pneumoniae*, and not only presented with hypopyon, but many ultimately perforated.

The main limitation of this study was that we only included moderate-to-severe ulcers, which limit the wider generalizability of the study findings. However, these ulcers are frequently the most difficult to manage, with the worse outcomes, therefore to identify the organisms involved and risk factors associated with such poor outcomes is an important step towards developing appropriate solutions for MK. Also, data was collected in a single institution that is a tertiary referral centre based in Madurai, South India. Although there is increasing urbanization of the villages/towns immediately surrounding Madurai, most of the villages in this region have remained rural, as indicated by census of India data.[18] Therefore, more severe cases may have presented to the Cornea Clinic at this institution, and the catchment area for the institution may include a greater rural population than other institutions.

In summary, in this study we have described several risk factors found in South Indian patients with moderate to severe MK, which include female gender, no formal education, and increased severity of the ulcer at presentation all associated with worse outcomes. Late-

stage MK resulted in severe visual impairment or blindness in half of the study participants by the final visit. Community-based prevention strategies aimed at women and those with low educational status might therefore improve visual outcomes by providing prophylactic antimicrobial treatment for corneal abrasions prior to the onset of MK.

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Author Contributions

Conception and design: JDC, NVP, MJB. Data acquisition: JDC, PS, SL, MS, PL, SE. Data analysis/interpretation: JDC, MJB. Manuscript preparation: JDC, MJB. Manuscript critical revision/approval of final version: all authors. All authors agree to be accountable for all aspects of the work and will ensure that any questions that may arise related to the accuracy or integrity of any part of the work will be appropriately investigated and resolved.

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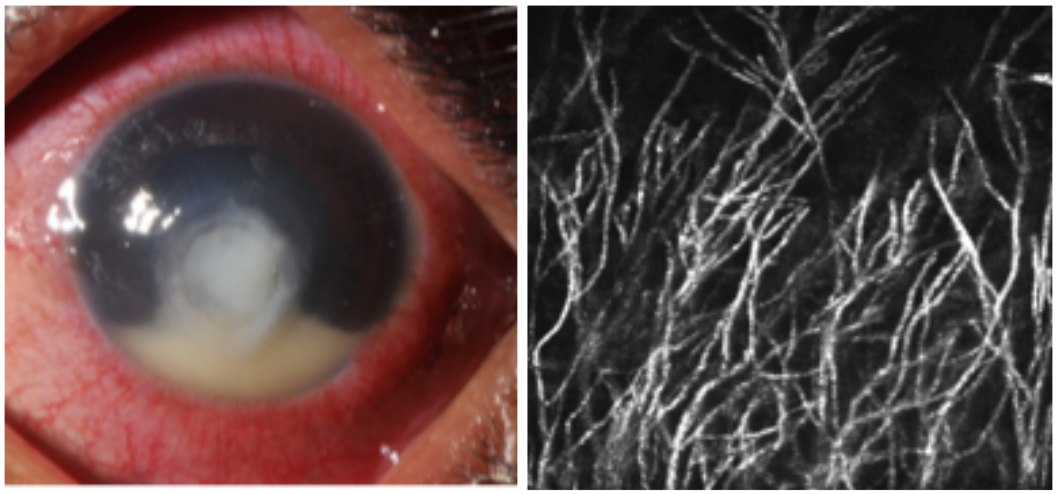
Chapter 4

Prospective Study of the Diagnostic Accuracy of the In Vivo Laser Scanning Confocal Microscope for Severe Microbial Keratitis



Clinical appearance of fungal keratitis and HRT3 *in vivo* confocal microscopy image of fungal filaments observed within the corneal stroma in fungal keratitis

4.1 Preamble

The manuscript in Chapter 4 describes the diagnostic accuracy of IVCM for the diagnosis of fungal and *Acanthamoeba* keratitis in the cohort study population, thus addressing the first PhD objective (i.e. objective 1a).

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Principal Supervisor	Matthew Burton
Thesis Title	Studies in the Diagnosis and Pathophysiology of Severe Microbial Keratitis

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SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I designed the study with assistance from co-authors Dr. Matthew Burton, Dr. Prajna and Dr. Larke. I recruited study participants (with help from Ms. Elakkiya & Dr. Shah), performed ulcer slit lam examinations and IVCM (along with co-authors Dr. P. Srikanthi & Dr. S. Lanjewar). I performed IVCM image grading, along with the other 4 graders (Mr. Hau, Dr. Vesaluoma, Dr. Carnt, Ms. Mason). I did the statistical analysis with guidance from Dr. Larke. I wrote the manuscript, and included edits as suggested by co-authors to form the final draft for publication.

Student Signature: [redacted] **Date: 8.8.18**

Supervisor Signature: [redacted] **Date: 8.8.18**

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Prospective Study of the Diagnostic Accuracy of the In Vivo Laser Scanning Confocal Microscope for Severe Microbial Keratitis

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

Design: Double-masked prospective cohort study.

Participants: Consecutive patients presenting to Aravind Eye Hospital, Madurai, India, between February 2012 and February 2013 with MK (diameter ≥3 mm, excluding descemetocele, perforation, or herpetic keratitis).

Methods: Following examination, the corneal ulcer was scanned by IVCM (HRT3/RCM, Heidelberg Engineering, Heidelberg, Germany). Images were graded for the presence or absence of fungal hyphae or *Acanthamoeba* cysts by the confocal microscopist who performed the scan (masked to microbial diagnosis) and 4 other experienced confocal graders (masked to clinical features and microbiology). The regrading of the shuffled image set was performed by 3 graders, 3 weeks later. Corneal-scrape samples were collected for microscopy and culture.

Main Outcome Measures: The main outcome measures were sensitivity, specificity, and positive and negative predictive values of IVCM compared with those of a reference standard of positive culture or light microscopy. Sensitivities and specificities for multiple graders were pooled and 95% confidence intervals calculated using a bivariate random-effects regression model.

Results: The study enrolled 239 patients with MK. Fungal infection was detected in 176 (74%) and *Acanthamoeba* in 17 (7%) by microbiological methods. IVCM had an overall pooled (5 graders) sensitivity of 85.7% (95% confidence interval [CI]: 82.2%–88.6%) and pooled specificity of 81.4% (95% CI: 76.0%–85.9%) for fungal filament detection. For *Acanthamoeba*, the pooled sensitivity was 88.2% (95% CI: 76.2%–94.6%) and pooled specificity was 98.2% (95% CI: 94.9%–99.3%). Intergrader agreement was good: κ was 0.88 for definite fungus; κ was 0.72 for definite *Acanthamoeba*. Intragrader repeatability was high for both definite fungus (κ: 0.88–0.95) and definite *Acanthamoeba* classification (κ: 0.63–0.90). IVCM images from 11 patients were considered by all 5 graders to have a specific organism present (10 fungus, 1 *Acanthamoeba*) but had negative results via culture and light microscopy.

Conclusions: Laser scanning IVCM performed with experienced confocal graders has high sensitivity, specificity, and test reproducibility for detecting fungal filaments and *Acanthamoeba* cysts in moderate to large corneal ulcers in India. This imaging modality was particularly useful for detecting organisms in deep ulcers in which culture and light microscopy results were negative. *Ophthalmology 2016;123:2285-2293 © 2016 by the American Academy of Ophthalmology. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).*

Supplemental material is available at www.aaojournal.org.

Severe microbial keratitis (MK) is an important cause of blindness worldwide.[1] In recent years, outbreaks of fungal and *Acanthamoeba* keratitis (AK) have brought to light the complexity of identifying a causative organism in these infections.[2] Although experienced cornea specialists can correctly identify fungal from bacterial keratitis based on clinical features alone in ≤66% of cases,[3] larger ulcers may present a diagnostic challenge, as tissue destruction may obscure classical features.[2] In these cases, microbiological techniques such as culture and light microscopy can aid in diagnosis but they do not offer a high diagnostic accuracy. Culture-positivity rates in MK vary widely, from 40% to

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73% in different settings, most likely because of the small size of corneal-scrape samples, prior antimicrobial treatment inhibiting microbial growth, and the fastidious nature of some organisms requiring special growth media (e.g., fungi and *Acanthamoeba*).[4–7] Direct visualization of fungal filaments or *Acanthamoeba* cysts in corneal scrapings using light microscopy can give a higher detection rate when compared with culture alone,[8] but it relies on the availability of trained, experienced observers who may not be present in some health care settings.

In vivo confocal microscopy (IVCM) is a noninvasive imaging technique that allows direct visualization of pathogens within the patient's cornea.[9] The 2 imaging modalities in current clinical use are the scanning slit IVCM (ConfoScan, Nidek Technologies, Fremont, CA) and the laser scanning IVCM (HRT3 with Rostock Corneal Module [RCM], Heidelberg Engineering, Heidelberg, Germany). The ConfoScan has a resolution of 1 micron laterally and up to 24 microns axially; the HRT3/RCM also has a lateral resolution of 1 micron but higher axial resolution of 7.6 microns.[10] Although many have reported the ability of both of these confocal microscopes to detect fungal filaments and *Acanthamoeba* cysts in human MK in vivo (summarized in Labbe et al[9]), only 2 studies have prospectively assessed the diagnostic accuracy of IVCM compared with standard microbiological techniques of culture with or without light microscopy.[11,12] Kanavi et al found that with a single IVCM grader, the ConfoScan 3.0 IVCM had a sensitivity of 100% for detection of *Acanthamoeba* and specificity of 84% compared with culture as the reference standard. For fungal filaments, the sensitivity was also high (94%) but the specificity lower (78%). The authors do not state whether the IVCM grader was masked to data from a clinical assessment of the patient. Vaddavalli et al also used the ConfoScan 3.0 with 2 IVCM graders who were masked to both the microbiological diagnosis and clinical assessment.[12] They found a sensitivity of 80% and specificity of 100% for the detection of *Acanthamoeba* cysts. For fungal filament detection, they found a sensitivity of 89.2% and specificity of 92.7%. In addition, a good interobserver agreement (κ 0.6) was found for the 2 graders. Hau et al have previously demonstrated that the diagnostic accuracy of IVCM for the diagnosis of MK is also affected by the experience of the IVCM grader.[13] As such, there is a need to determine the extent of variability between graders in the clinical setting. Resolution of the IVCM imaging system may also affect the ability of graders to detect pathogens, but to date there have been no formal prospective studies using the higher resolution HRT3 IVCM in the detection of MK.

In this study, we aimed to determine the diagnostic accuracy of HRT3 IVCM in moderate to severe MK in South India using 5 experienced confocal graders (masked to microbiological diagnosis). We also assessed intergrader and intragrader agreement.

Methods

This study was approved by the institutional review board of Aravind Eye Hospital, Tamil Nadu, India; the Indian Council for Medical Research; and the Ethics Committee of the London School of Hygiene and Tropical Medicine. Prior to enrollment in the study, all patients gave written informed consent; study participants who were illiterate gave informed consent with a witnessed thumbprint on the study consent form, as approved by the above ethics committees. This study adhered to the tenets of the Declaration of Helsinki and was conducted in accordance with the Standards for Reporting of Diagnostic Accuracy studies (STARD)[14]—see the STARD checklist in Supplementary Table S1, available at aaojournal.org.

Study Participants

This study was based in the Cornea Clinic at Aravind Eye Hospital, Madurai, Tamil Nadu, India. Consecutive patients presenting to the clinic between February 2012 and February 2013 were assessed for eligibility and prospectively enrolled into the study if they were found eligible. The inclusion criteria were age ≥18 years and the presence of a large corneal ulcer, defined as a stromal infiltrate ≥3 mm at the longest diameter, with an overlying epithelial defect and signs of acute inflammation. All eligible patients underwent slit lamp examination by an ophthalmologist (cornea specialist), and relevant clinical history and examination findings were recorded in the standardized study form. We excluded any patients with a descemetocele or >80% corneal thinning in the affected eye as assessed on slit lamp examination (i.e., in whom we could not safely applanate the IVCM onto the cornea for imaging), those considered to have herpetic stromal keratitis on clinical grounds (i.e., either a prior history of the disease or the presence of clinical features associated with herpetic disease), or if Snellen visual acuity was worse than 6/60 in the unaffected eye.

IVCM Imaging

The affected eye was anesthetized using 0.5% proparacaine eye drops (Aurocaine, Aurolab, Madurai, India), and volume scans of the corneal ulcer were obtained using the HRT3 IVCM (Heidelberg Engineering, Heidelberg, Germany) with RCM (63× magnification objective lens, Nikon, Tokyo, Japan), by an ophthalmologist trained in performing IVCM and following a standard procedure described elsewhere.[13] Briefly, volume scans were obtained in the center of the ulcer, and at the 12-, 3-, 6-, and 9-o'clock positions of the peripheral ulcer margins. Volume scans were taken from the surface of the ulcer and manually refocused several times to take progressively deeper overlapping scan sets covering as much of the full depth of the ulcer as possible.

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

Microbiological Diagnosis

Immediately after IVCM had been performed and grading recorded, the base and leading edge of the corneal ulcer were scraped using a flame-sterilized Kimura spatula. Scrapings were immediately placed onto 2 glass slides for light microscopy and on agar plates for culture: blood agar (BA), potato dextrose agar (PDA), and nonnutrient agar seeded with *Escherichia coli* in the laboratory if AK was clinically suspected. Standard microbiological methods were followed to detect any pathogen.[15] In brief, slides were stained with 10% potassium hydroxide or gram or Giemsa stain to aid in the visualization of fungal filaments, bacteria, or *Acanthamoeba* cysts,

respectively; agar plates were incubated at 37° C for 2 days for BA or at 27° C for 7 days for PDA and were assessed daily for organism growth. A culture was classified as positive if any of the following criteria were satisfied: (a) growth of the same species of bacteria or fungus on at least 2 solid media, or (b) semiconfluent growth at the site of inoculation in 1 solid medium of an organism that, for bacteria, was the same as the organism identified with gram stain on microscopy. Organism speciation was performed using standard laboratory methods.[15] For fungal identification, spores were stained with lactophenol cotton blue and speciated by the morphological appearance of the colony, hyphae, and spores.[16]

IVCM Grading

Patient-identifying data were removed from all IVCM scans, and images were arranged in a random order for each observer to assess. At Moorfields Eye Hospital, our confocal graders assessed all scans of all recruited patients and graded for the definite presence, definite absence, or possible presence of fungal filaments or *Acanthamoeba* cysts as described above for grader 5. All graders had varying experience of performing IVCM and grading confocal images for MK, ranging from 6 years (graders 1 and 2; grader 2 with an additional 2 years of general IVCM experience), to 3.5 years (grader 3), to 2 years (graders 4 and 5, specifically with IVCM MK imaging experience). All graders were masked to the microbiological diagnosis. Graders 1 to 4 were masked to the clinical appearance of the ulcer. Grading data were directly entered into a Microsoft Access 2010 database. To measure intragrader agreement, all image sets were allocated a new random study number and shuffled into a new order. Three graders were able to repeat the grading process at least 3 weeks after the first grading session.

Reference Standard

For the purposes of this study, the reference for diagnosis of fungus was a positive culture or (if the culture was negative) the presence of fungal hyphae on light microscopy, as has been used in previous studies.[17] Similarly, the reference for *Acanthamoeba* was a positive culture or presence of *Acanthamoeba* cysts on light microscopy; this approach has previously been shown to increase diagnostic accuracy for *Acanthamoeba* detection compared with the use of culture alone.[18] One experienced microbiologist performed the culture and light microscopy interpretation and was masked to the IVCM images and grading but had access to a limited clinical history that was available on the microbiology test request form.

Statistical Methods

All statistical analyses were performed in Stata version 12.1 (StataCorp, College Station, TX). Sample size was estimated as n = 200 based on a fungal keratitis prevalence estimate of 50%, aiming for a sensitivity of 85%, and with marginal error of 7%, in accordance with Hajjan-Tilaki et al.[19] Statistical significance of between-group differences in demographic or clinical features was assessed using the Kruskal–Wallis test and chi-square test for proportions. Sensitivity (i.e., ratio of true-positives/true-positives plus false-negatives), specificity (i.e., ratio of true-negatives/true-negatives plus false-positives), positive predictive value, and negative predictive value were calculated using definite fungus or definite *Acanthamoeba* grades for the primary analysis. The primary outcome measure was the pooled sensitivity and specificity of the 5 graders, calculated along with 95% confidence intervals using a bivariate random-effects regression model that accounts for the correlation between the 2 measures (*metandi* and *midas* commands in Stata).[20–22] This is likely to be a conservative estimate because it accounts for the various levels of experience of the graders and only 1 grader takes into account the clinical features of the ulcer. Comparison of regraded outcomes with initial grades was performed using the κ score to calculate intragrader agreement (to assess reproducibility). A κ score was also calculated for intergrader agreement (to assess reliability) for cases graded with certainty as definite fungus/*Acanthamoeba* or no organism seen. The κ scores were interpreted as follows: ≤0.20 *no* agreement; 0.2 to 0.39 minimal agreement; 0.40 to 0.59 weak agreement; 0.60 to 0.79 moderate agreement; 0.80 to 0.90 strong agreement; >0.90 almost perfect agreement.[23]

Results

Study Participants

A total of 254 patients were assessed for study eligibility between February 2012 and February 2013, of whom 13 patients were excluded for history of herpetic keratitis (n = 1) or presence of >80% corneal thinning (n = 12). Two patients were also excluded as we were unable to perform diagnostic tests for them: no culture or light microscopy performed (n = 1, deep stromal abscess), or total ulcer with no clear cornea to scan with IVCM (n = 1)—see Supplementary Figure S1 for STARD patient flow diagram, available at www.aaojournal.org. A total of 3163 volume scans were obtained with a mean 13 volume scans per patient (range 3–42 scans). A few patients (n = 4) were unable to cooperate for the full IVCM imaging protocol, and so we were only able to image part of the ulcer—these patients were not excluded. No adverse events were noted from either performing IVCM imaging or corneal scraping for culture or light microscopy.

Sociodemographic features of the final participants are shown in Table 1. Compared with all others, AK patients had a higher frequency of ring infiltrate (88% in AK vs. 31% all others, $P < 0.0001$) and a longer median symptom duration (30 days in AK vs. 7 days all others, $P < 0.0001$).

Microbiological Culture and Light Microscopy Results

Tables 2 and 3 summarize the organisms identified on microbiological testing in the 239 patients included in the analysis. Most patients (74%, n = 176) met the reference standard criteria of fungal positivity. These included 2 cases of mixed infection, i.e., fungal filaments detected on light microscopy but positive culture for bacteria (*Streptococcus viridans* and *Streptococcus pneumoniae,* respectively). Thirty participants had fungal filaments detected on light microscopy alone (negative culture for fungus), of whom 83% (n = 25) had used antifungal therapy prior to presentation and 50% (n = 15) were deep with the stromal infiltrate involving the posterior third of the cornea. All 17 *Acanthamoeba* cases were culture positive, and 13 of these were also light microscopy positive (none was solely light microscopy positive for *Acanthamoeba*). The culture-positivity rate for any organism was high, at 76% (n = 182).

Detection of Fungal Filaments by IVCM

Figures 1A and B show an example of fungal filaments as seen in IVCM images of a culture-positive fungal ulcer. Overall, all 5 graders were able to definitely detect fungal filaments in the IVCM images, with a pooled sensitivity of 85.7% (95% confidence interval [CI]: 82.2%–88.6%) and pooled specificity of 81.4% (95% CI: 76.0%–85.9%), with individual grader data shown in Table 4A. Overall, the highest sensitivity (89.8%, 95% CI: 84.3%–93.8%) was achieved by the grader with access to the ulcer clinical

Table 1. Demographic Data and Clinical Features of Study Participants

	Fungal Keratitis (74%, n = 176)*	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 (%)†	112 (72.3)	14 (87.5)	13 (81.3)	14 (63.6)	0.3509
Prior antifungal use, n (%)†	89 (57.4)	10 (62.5)	7 (43.8)	13 (59.1)	0.7965
No. of patients with ring infiltrate, n (%)	52 (29.6)	15 (88.2)	10 (52.6)	7 (25.9)	0.0001

*Mixed infections included culture positive for bacteria but microscopy positive for fungus, n = 2.
†For prior drug use, n = 209 (data not available for 30 patients).

features (grader 5). The grader with the lowest sensitivity (grader 2, 79.1%) also had the highest specificity (i.e., fewest false-positives). For only the 4 graders who were masked to clinical features, pooled sensitivity was 84.5% (95% CI: 80.8%–87.6%) and pooled specificity was 82.0% (95% CI: 75.7%–86.9%). Cases with earlier presentation and shorter symptom duration (≤4 days) had the highest pooled sensitivity for all 5 graders, 95% (95% CI: 88–98%), but the lowest pooled specificity, 53% (95% CI: 39%–66%). As symptom duration increased to >10 days, the pooled sensitivity reduced to 72% (95% CI: 64%–78%), with a concomitant increase in sensitivity, to 91% (95% CI: 84%–95%), as shown in Table 5.

There was a strong intergrader agreement among all 5 masked graders' scores for definite fungus, with a κ score of 0.88 ($P < 0.0001$). The κ scores for intragrader agreement (i.e., test reproducibility) were between 0.88 and 0.95 ($P < 0.0001$), i.e., strong to almost perfect agreement.

IVCM images for the 3 culture-positive *Nocardia* spp. cases were classified by 4 of the 5 graders as not having filamentous structures.

IVCM False-Positives or False-Negatives for Fungus

Ten patients were microbiologically negative for fungus, but ≥4 graders categorized these images as showing definite fungus (i.e., IVCM false-positives). Figure 2 shows examples of the fungal branching structures seen in these IVCM images. Of these 10 ulcers, 9 were noted to be deep with extension into the posterior third of the cornea on slit lamp examination or IVCM imaging.

Table 2. Distribution of Organisms Identified by Culture or Light Microscopy

	N	%
Culture positive (n = 182)		
Acanthamoeba	17	7.1
Fungi	144	60.3
Bacteria	19	9.6
Mixed: Culture positive for bacteria, microscopy positive for fungi	2	0.8
Culture negative (n = 57)		
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
Total	239	100

Conversely, 9 patients were microbiologically positive for fungus but graded by all 5 graders as having no fungal filaments on IVCM (i.e., IVCM false-negatives). On further IVCM imaging ≤21 days after presentation, fungal filaments were still not detected in 5 patients and the remaining 4 patients had progressive corneal thinning or perforation that prevented further IVCM imaging from being performed. Five patients had surface plaques at presentation that caused high reflectivity and difficulty in imaging the ulcer clearly using IVCM. The spectrum of organisms grown from the IVCM false-negative ulcers included *Fusarium* sp. (n = 4), *Aspergillus* sp. (n = 3), *Cylindrocarpon* sp. (n = 1); in 1 patient, no organism was grown but fungal filaments were detected in corneal scrapings on light microscopy.

IVCM Detection of *Acanthamoeba* Cysts

For definite detection of *Acanthamoeba* cysts, the 5 graders had a pooled sensitivity of 88.2% (95% CI: 76.2%–94.6%) and pooled specificity of 98.1% (95% CI: 94.9%–99.3%). The 4 graders masked to clinical features had a very similar pooled sensitivity, 88.5% (95% CI: 73.0%–95.6%), and pooled specificity, 98.0% (95% CI: 93.3%–99.4%). The grader with access to clinical-feature data had a sensitivity of 88.2% and specificity of 98.6% (grader 5, Table 4B). In ulcers presenting earlier (i.e., <20 days symptom duration), the pooled sensitivity and specificity (all 5 graders) were 82% (95% CI:34%–98%) and 98% (95% CI:

Table 3. Species Cultured for Fungi (n = 144) and Bacteria (n = 21)

Organism	Species	N	%
Fungi: hyaline	*Fusarium* sp.	73	50.7
	Aspergillus flavus	26	18.1
	Aspergillus fumigatus	5	3.5
	Aspergillus terreus	2	1.4
	Cylindrocarpon sp.	1	0.7
	Unidentified hyaline fungi	14	9.7
Fungi: dematiaceous	*Curvularia* sp.	5	3.5
	Exserohilum sp.	4	2.8
	Lasiodiplodia sp.	2	1.4
	Bipolaris sp.	1	0.7
	Unidentified dematiaceous fungi	11	7.6
Bacteria: gram-positive	*Streptococcus pneumoniae*	10	47.6
	Streptococcus viridans	3	14.3
	Staphylococcus epidermidis	2	9.5
	Nocardia sp.	3	14.3
Bacteria: gram-negative	*Pseudomonas aeruginosa*	2	9.5
	Aeromonas sp.	1	4.8

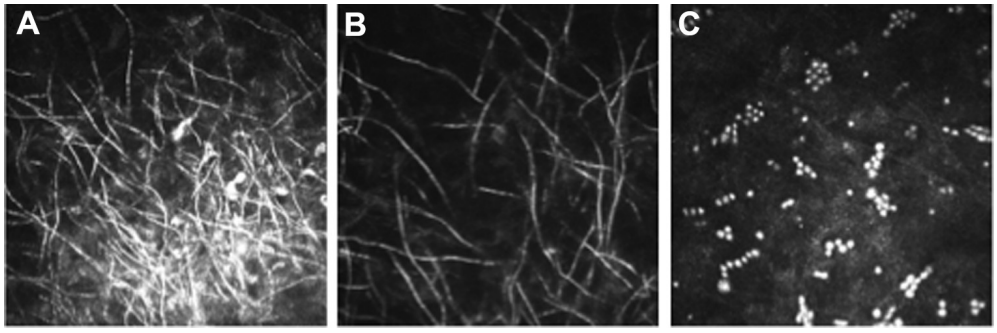

Figure 1. In vivo confocal microscopy (IVCM) images of *Fusarium* sp. culture-positive ulcer showing **A,** overlapping fungal filaments in the center of the ulcer and **B,** more-distinct fungal filaments at the periphery; **C,** IVCM images of an *Acanthamoeba* sp. culture-positive ulcer showing cysts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

95–99%), respectively. This high sensitivity and specificity was maintained in ulcers with symptom duration >30 days (Table 5).

For all 5 graders, there was a moderate intergrader agreement, with a κ score of 0.72 ($P < 0.0001$). The κ scores for intragrader agreement for definite *Acanthamoeba* cases ranged from 0.63 to 0.90 ($P < 0.0001$). *Acanthamoeba* cyst morphology is shown in Figure 1C.

IVCM False-Positives or False-Negatives for *Acanthamoeba*

In the 1 IVCM false-positive case, culture and light microscopy results were both negative for *Acanthamoeba*, but all 5 graders detected *Acanthamoeba* cysts on IVCM. Figure 2F shows images from this patient highlighting the presence of *Acanthamoeba* cystlike structures.

There was 1 IVCM false-negative ulcer, i.e., microbiologically positive for *Acanthamoeba* sp. but no definite *Acanthamoeba* detected by any grader. Of note, 2 of the 5 graders classified the images for this ulcer as possible *Acanthamoeba*.

Possible Fungus or *Acanthamoeba* on IVCM

In total, 71 ulcers were classified as possible fungus present by any grader, with agreement from ≥3 graders on this diagnosis in 7 of these ulcers. The reference standard was fungal positive in 75.3% (n = 55) of those graded as possible fungus. The remainder either had no growth with no organism on light microscopy (n = 9) or were culture or light microscopy positive for *Acanthamoeba* sp. (n = 3), *Nocardia* sp. (n = 2), or *S. pneumoniae* (n = 2).

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

At regrading, ≤57% of all images initially classified by any grader as possible fungus were shifted to the definite fungus category (n = 34/60), and 85% of these were positive for fungus according to the reference standard (n = 29/34). Of the images initially graded as possible *Acanthamoeba*, 9% (n = 8/88) were shifted to the definite *Acanthamoeba* grade at regrading, with 75% (n = 6/8) of these being microbiologically positive for *Acanthamoeba*. Very few images were converted by any grader from definite fungus to possible fungus (n = 11/438). Of these images, 6 were converted by at least 2 of the 3 graders (*Curvularia* sp. n = 2; *Fusarium* sp. n = 2; culture/light microscopy negative, n = 2), and the remaining images were culture positive for *Aspergillus flavus* (n = 2), *Fusarium* sp. (n = 1), *Nocardia* sp. (n = 1), or culture/light microscopy negative (n = 1). For *Acanthamoeba*, again few images were regraded from definite to possible (n = 9/58), with 8 images converted by at least 2 of 3 graders (4 culture positive for *Acanthamoeba* sp., 2 for *Fusarium* sp., 2 for *Nocardia* sp.), and the remaining 1 culture positive for *Fusarium* sp.

Discussion

Large corneal ulcers can present a major diagnostic challenge, especially as they often have mixed or atypical clinical features and may be culture negative. Delays in treatment of fungal keratitis and AK in particular can lead to

Table 4A. Sensitivity, Specificity, Positive Predictive Value (PPV), and Negative Predictive Value (NPV) for Definite Detection of Fungi on IVCM Compared with Culture or Light Microscopy

Grader	N*	TP	TN	FP	FN	Sensitivity (%, 95% CI)	Specificity (%, 95% CI)	PPV (%, 95% CI)	NPV (%, 95% CI)
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†	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)

FN = false-negative; FP = false-positive; TN = true-negative; TP = true-positive.
*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).
†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 IVCM Compared with That of Culture 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†	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)

FN = false-negative; FP = false-positive; TN = true-negative; TP = true-positive.
*The total number 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.
†Grader 5 was unmasked to ulcer clinical features.

significant visual loss, and even loss of the eye.[5,24,25] IVCM is a noninvasive method through which fungal filaments and *Acanthamoeba* cysts can be immediately detected in the patient's cornea,[9] allowing the clinician to promptly start the correct antimicrobial therapy. In 2004, the American Academy of Ophthalmology conducted an evidence-based assessment of the value of IVCM as a diagnostic tool for MK. With only level II and III evidence available at that time, they concluded that IVCM could be useful as an adjunctive test in the diagnosis of fungal keratitis, but for AK there was sufficient evidence to support the use of IVCM as the sole diagnostic test.[26] Since then, 2 prospective studies using the ConfoScan IVCM have found a high sensitivity and specificity for the detection of fungal filaments and *Acanthamoeba* cysts.[11,12] To the best of our knowledge, this is the first report of the high diagnostic accuracy of the HRT3 confocal microscope in the detection of fungi and *Acanthamoeba* in moderate to severe MK in a clinical setting, comparable to the results found in these previous 2 studies. The use of a multigrader approach allowed for a more accurate assessment of sensitivity and specificity. Our study demonstrated a slightly higher sensitivity for detection of *Acanthamoeba* than fungal filaments compared with that found in the study by Vaddavalli et al. We were able to study only a small number of participants with AK, and so further research is required with a larger study population, as well as with patients in earlier stages of disease, to more fully evaluate the use of HRT3 IVCM for the diagnosis of AK.

Table 5. Pooled Sensitivity and Specificity for All 5 Graders by Symptom Duration (Split by Quartile)

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)

We found that experienced IVCM graders were able to detect fungi or *Acanthamoeba* in 94.8% of all culture-positive or light microscopy-positive ulcers. The main cause of IVCM false-negatives was technical difficulty in being able to obtain adequate IVCM images. Ulcers with superficial plaques caused a high level of surface reflectivity in the IVCM images, thus inhibiting recognition of fungal filaments in the ulcer surface or margins, as we found in 5 of our 9 IVCM false-negative fungal ulcers. Some patients were able to tolerate IVCM imaging for only a short time period and so only a limited number of images were obtained, and these images may not have captured pathogens present in deeper aspects of the ulcer. False-negatives due to poor patient cooperation have been previously reported with this imaging modality.[27] In the case of our 11 IVCM false-negatives, the clinical features as well as microbiological results in these patients were able to guide appropriate treatment. Other reasons for IVCM false-negatives include the learning curve for the IVCM operator in adequately scanning the entire ulcer to capture any pathogen in the images, as well as the presence of a high degree of stromal inflammation that could mask the presence of the pathogen (i.e., through high reflectivity that reduces image contrast as with surface plaques or difficulty in identifying *Acanthamoeba* cysts in the presence of many white cells because they both have similar morphology).

We found that IVCM graders were able to detect a pathogen in 11 culture-negative and light microscopy–negative ulcers. The IVCM images in these ulcers had classical features of fungal hyphae or *Acanthamoeba* cysts, and so we believe these represent true cases of disease. In most patients, these ulcers were deep, involving the posterior third of the cornea and therefore making it less likely that superficial corneal scraping would collect viable fungi to grow in culture or to be seen via light microscopy. In such cases, IVCM is an invaluable tool to rapidly detect fungal filaments in the deep stroma, and it allows the correct antimicrobial treatment to be commenced without the need for an invasive corneal biopsy to identify the pathogen.[28] Other causes of a false-positive IVCM for fungus include the presence of other linear branching structures such as corneal nerves and *Nocardia* sp. filaments.[29] Only 1 grader out of 5 classified images from *Nocardia* keratitis as containing fungal filaments in this study. Because *Nocardia* sp. filaments are thinner in diameter than filamentous fungi

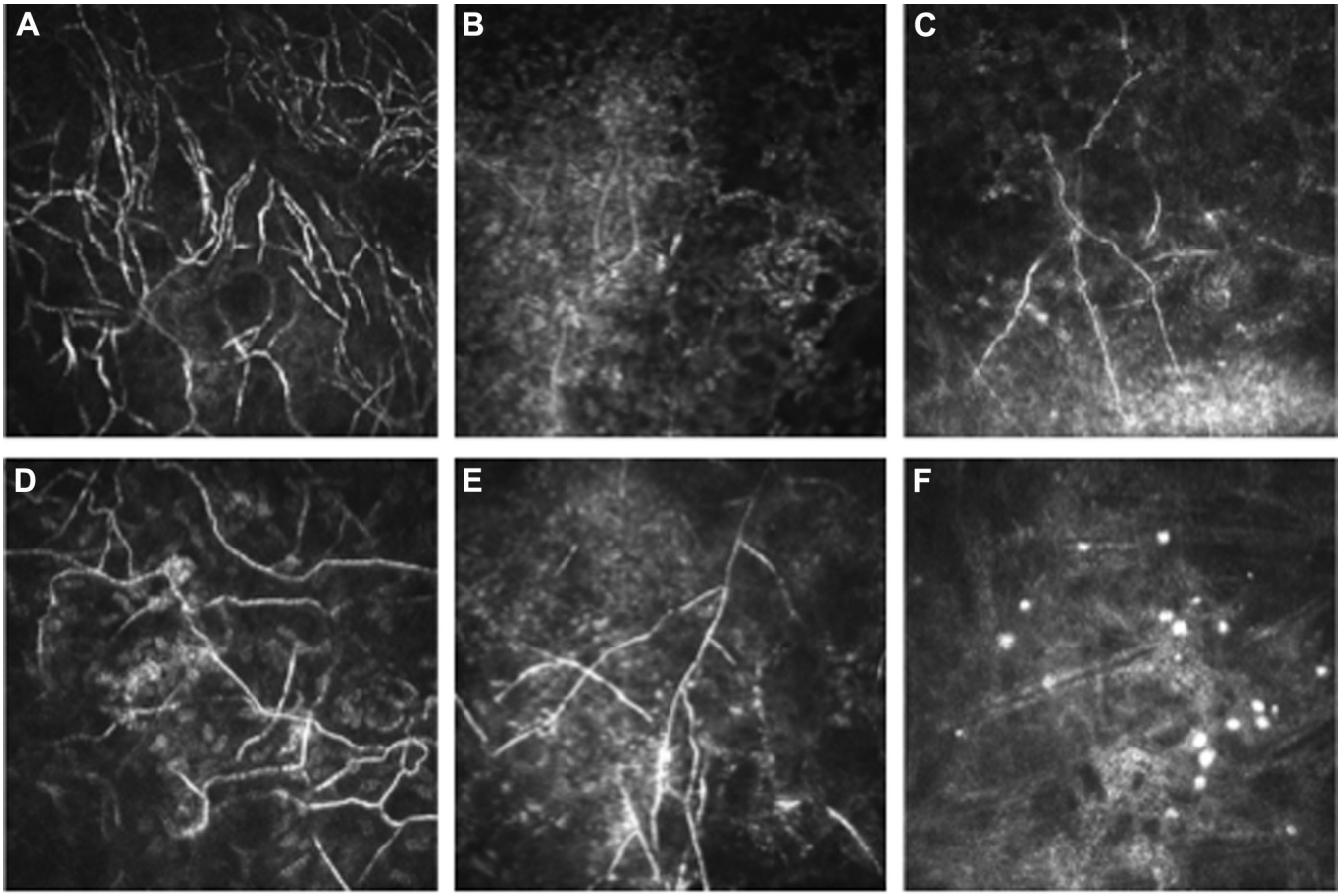

Figure 2. In vivo confocal microscopy images of 6 culture-negative and light microscopy-negative ulcers in which graders detected fungal filaments **A**–**E**, or Acanthamoeba cysts (**F**). Note the similarity of cyst appearance to those in Figure 1 image **C** with a similar absence of inflammatory cell infiltrate in the corneal stroma.

(<1.5 microns vs. 3-6 microns, respectively),[30] they can be more difficult to detect on IVCM images, particularly in the presence of significant stromal edema or inflammation, as in moderate to severe keratitis, but they were readily detected microbiologically in our study.

In the clinical setting, an uncertain IVCM test result can cause concern with regard to which antimicrobial therapy to commence. On further analysis of all images graded as showing the possible presence of a pathogen, 75% of those graded as possible fungus were appropriately classified when compared with the reference standard, but <10% of the images graded as possible *Acanthamoeba* corresponded to microbiologically confirmed acanthamoebal ulcers. This finding confirms the importance of adding clinical examination and microbiological testing to IVCM imaging to reach a definite diagnosis for acanthamoebal infection in particular, rather than using 1 diagnostic tool alone, as also found by other investigators.[18]

There was an apparent improvement in the certainty of diagnosis on regrading images. This learning effect was also detected by Hau et al, who found that the specificity improved for all graders on IVCM MK image regrading at a later date.[13] They also found that, as the level of IVCM experience of the grader increased,[13] the diagnostic accuracy for detection of MK also improved, thus indicating the importance of training in IVCM image recognition for all new graders. The IVCM grader may also benefit from having access to a clinical image of the ulcer,[18] because our grader with access to clinical-feature information had a higher sensitivity for fungal detection.

In this study, although the graders were from a variety of backgrounds (ophthalmic nurses, optometrists, and ophthalmologists) and levels of experience, they had a high intergrader agreement for pathogen detection. We found higher κ scores for intergrader agreement than Vaddavalli et al found,[12] a difference that may be due to the higher resolution of the HRT3 imaging system allowing for higher-definition images of the pathogen, as well as the training or experience of our confocal graders with this high-resolution imaging system. Intraobserver agreement in our study was also high, and it was better for fungal detection, with the best agreement in the most experienced observer.

Limitations of this study include the dominance of filamentary fungal keratitis and the relatively low proportion of bacterial infections. We were unable to study confocal appearances of candida keratitis, which is more common in more temperate climates. We studied only 17 cases of *Acanthamoeba*, and so further research is needed to more fully elucidate acanthamoebal detectability on IVCM

imaging in a larger study. The cost of the confocal microscope may be too high for its routine uptake in areas with the highest endemicity for fungal keratitis and AK, in low- and middle-income countries in tropical regions; however, delay in treatment may result in a greater cost in the long-term because of poorer visual outcome related to delayed diagnosis.

There was a high culture-positive rate in this study. We believe there are a number of reasons for this, in addition to our inclusion of mainly larger ulcers. First, we used a microbiology service that is particularly optimized for ocular microbiology. Second, a culture could be initiated with very little delay after sample collection because the laboratory is situated next to the Cornea Clinic at Aravind Eye Hospital. Third, the standard practice is to use a Kimura spatula, which we also believe gives a more ample sample than using a needle does, thereby improving the organism detection rate. In regions with lower culture-positivity rates, the value of IVCM may be greater, as a higher proportion of cases will be culture negative. Although our study has focused on larger ulcers, we still found that IVCM can detect fungi with a high sensitivity in ulcers with only a few days' symptom duration. Also, for *Acanthamoeba* detection with IVCM, we found a high sensitivity and specificity for both early- and late-presenting ulcers.

In summary, we have found that experienced graders are able to detect fungal or acanthamoebal elements within HRT3 IVCM images with high sensitivity, specificity, and test reproducibility in moderate to severe keratitis. This imaging modality outperforms standard microbiological methods for deep ulcers in particular. The addition of clinical-feature data improved diagnostic accuracy. IVCM may therefore be considered an adjunctive tool, in addition to clinical examination and microbiological testing, for detection of fungi or *Acanthamoeba* in MK.

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Analysis and interpretation: Chidambaram Prajna, Larke, Carnt, Vesaluoma, Hau, Burton

Data collection: Chidambaram, Prajna, Palepu, Lanjewar, Shah, Elakkiya, Lalitha, Carnt, Vesaluoma, Mason, Hau, Burton

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Overall responsibility: Chidambaram, Prajna, Larke, Palepu, Lanjewar, Shah, Elakkiya, Lalitha, Carnt, Vesaluoma, Mason, Hau, Burton

Abbreviations and Acronyms:
AK = *Acanthamoeba* keratitis; **BA** = blood agar; **CI** = confidence interval; **IVCM** = in vivo confocal microscopy; **MK** = microbial keratitis; **NPV** = negative predictive value; **PDA** = potato dextrose agar; **PPV** = positive predictive value.

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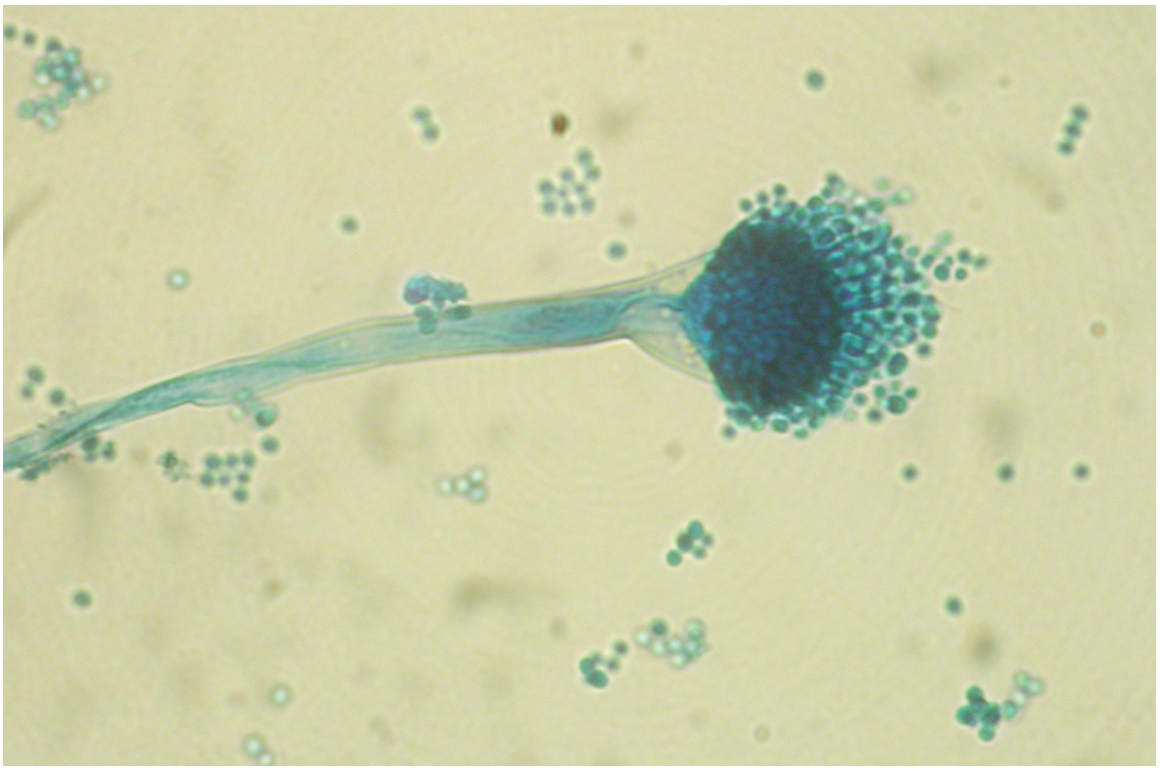
Chapter 5

In vivo confocal microscopy appearance of *Fusarium* and *Aspergillus* species in fungal keratitis



Aspergillus flavus fungal hyphae and spores stained with Lactophenol cotton blue, grown from a corneal scraping from fungal keratitis in Aravind Eye Hospital, Madurai

5.1 Preamble

The manuscript in Chapter 5 describes the morphological differences of *Fusarium* and *Aspergillus* keratitis, in particular fungal filament branching angle, as observed with IVCM.

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Student	Jaya Chidambaram
Principal Supervisor	Matthew Burton
Thesis Title	Studies in the Diagnosis and Pathophysiology of Severe Microbial Keratitis

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	British Journal of Ophthalmology		
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SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I designed the study with assistance from co-authors Dr. Matthew Burton, Dr. Prajna and Dr. Larke. I recruited study participants (with help from Ms. Elakkiya & Dr. Shah), performed ulcer slit lamp examinations and IVCM (along with co-authors Dr. P. Srikanthi & Dr. S. Lanjewar). I performed all of the fungal image analysis. I analysed the data with guidance from Dr. Larke. I wrote the manuscript, and included edits as suggested by co-authors to form the final draft for publication.

Student Signature: [redacted] **Date: 8.8.18**

Supervisor Signature [redacted] **Date: 8.8.18**

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In vivo confocal microscopy appearance of *Fusarium* and *Aspergillus* species in fungal keratitis

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ABSTRACT

Background Clinical outcomes in fungal keratitis vary between *Fusarium* and *Aspergillus* spp, therefore distinguishing between species using morphological features such as filament branching angles, sporulation along filaments (adventitious sporulation) or dichotomous branching may be useful. In this study, we assessed these three features within Heidelberg Retina Tomograph 3 in vivo confocal microscopy (IVCM) images from culture-positive *Fusarium* and *Aspergillus* spp keratitis participants.

Methods Prospective observational cohort study in Aravind Eye Hospital (February 2011–February 2012). Eligibility criteria: age ≥18 years, stromal infiltrate ≥3 mm diameter, *Fusarium* or *Aspergillus* spp culture-positive. Exclusion criteria: previous/current herpetic keratitis, visual acuity <6/60 in fellow eye, >80% corneal thinning. IVCM was performed and images analysed for branch angle, presence/absence of adventitious sporulation or dichotomous branching by a grader masked to the microbiological diagnosis.

Results 98 participants were included (106 eligible, 8 excluded as no measurable branch angles); 68 were positive for *Fusarium* spp, 30 for *Aspergillus* spp. Mean branch angle for *Fusarium* spp was 59.7° (95% CI 57.7° to 61.8°), and for *Aspergillus* spp was 63.3° (95% CI 60.8° to 65.8°), p=0.07. No adventitious sporulation was detected in *Fusarium* spp ulcers. Dichotomous branching was detected in 11 ulcers (7 *Aspergillus* spp, 4 *Fusarium* spp).

Conclusions There was very little difference in the branching angle of *Fusarium* and *Aspergillus* spp. Adventitious sporulation was not detected and dichotomous branching was infrequently seen. Although IVCM remains a valuable tool to detect fungal filaments in fungal keratitis, it cannot be used to distinguish *Fusarium* from *Aspergillus* spp and culture remains essential to determine fungal species.

INTRODUCTION

Fungal keratitis is increasing in incidence throughout the world.[1] In warm, humid climates such as South India, filamentous fungi are the cause of more than 60% of fungal corneal ulcers, with *Fusarium* and *Aspergillus* being the two predominant species.[2] There is evidence that corneal healing and visual outcomes can be very different for keratitis caused by these two species; corneal infection with *Aspergillus* spp is associated with slower re-epithelialisation, increased risk of perforation and worse visual acuity at 3 months after presentation compared with *Fusarium* spp.[3] This may in part be due to the greater susceptibility of *Fusarium* spp to natamycin, which is the main antifungal agent used to treat filamentous fungal keratitis.[4]

At present, microbiological culture of corneal scrapings remains the standard method of identifying the causative organism in fungal keratitis. Morphological features of fungal colonies and spores allow accurate identification of the fungal species, but require an experienced microbiologist and can take up to 7 days for fungi to grow and sporulate in culture.[5] In contrast, the high-resolution imaging modality of in vivo confocal microscopy (IVCM) provides immediate visualisation of filamentous fungi within the living cornea.[6] Previous case reports have described the IVCM appearances of filaments or hyphae of *Aspergillus* spp in patients as well as animal models of keratitis; *Aspergillus fumigatus* hyphae were reported to measure up to 3–10 microns in diameter, 200–400 microns in length and have hyphal branches emerging at 45° from the parent hyphae.[6 7] Some have postulated that the branching patterns of fungi as seen in IVCM images of keratitis could be used to differentiate fungal species. Brasnu *et al*[8] analysed IVCM images of keratitis obtained using the Heidelberg Retina Tomograph (HRT)II confocal microscope (Heidelberg Engineering, Heidelberg, Germany) in four patients with *Fusarium* spp infection, and donor corneas infected in vitro with *A. fumigatus* and *Fusarium solani*. *Fusarium* spp filaments were reported to be 3–5 microns in diameter, 200–300 microns in length, with a hyphal branching angle of 90° in IVCM images from patients and from the infected donor cornea. For *A. fumigatus*, the branching angle in the infected donor cornea was measured as 45°.[8] In contrast to these IVCM findings, reports of histopathological examination of fungal hyphae in tissue sections show that acute angle branching may occur in both *Fusarium* and *Aspergillus* spp.[9] Other morphological traits have also been proposed as potential diagnostic features of certain fungi in histopathology, for example, presence of spores along hyphae (known as adventitious sporulation) in tissue samples from *Fusarium* keratitis.[10] Also, in *Aspergillus* spp, the apical filament can directly bifurcate instead of generating side branches—this is known as dichotomous branching.[9] To our knowledge, these features have not as yet been studied in IVCM images of fungal keratitis.

In this study, we investigated whether hyphal branching angles measured using IVCM images

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from patients with fungal keratitis differed between culture-positive *Fusarium* and *Aspergillus* spp corneal ulcers, and also assessed whether adventitious sporulation or dichotomous branching could be detected using IVCM.

MATERIALS AND METHODS

This study was approved by the Indian Council of Medical Research, as well as the Ethics committees of Aravind Eye Care System, India and the London School of Hygiene and Tropical Medicine, UK. Tenets of the Declaration of Helsinki were adhered to in conduct of this study. Participants were enrolled after they had given their written informed consent, or a thumbprint to indicate consent in illiterate participants, witnessed by a study team member, as approved by the ethics committees.

Study participants

Patients with clinically suspected microbial keratitis presenting to the Cornea Clinic at Aravind Eye Hospital, Madurai, Tamil Nadu (India) between February 2011 and February 2012 were assessed for eligibility to enter the study. Eligibility criteria were: age ≥18 years, corneal ulcer measuring ≥3 mm in longest diameter of stromal infiltrate and extending ≥1/3 of the corneal thickness, with an overlying epithelial defect and evidence of acute inflammation (ie, conjunctival injection and/or either anterior chamber cells, flare or hypopyon). Exclusion criteria were: previous or current herpetic keratitis, fellow eye visual acuity <6/60, descemetocele or >80% corneal thinning as assessed on slit lamp examination (due to inability to safely applanate the cornea for IVCM).

In vivo confocal microscopy

The corneal ulcer was imaged according to a standard protocol, as previously described.[11] The HRT3 laser scanning confocal microscope with Rostock Corneal Module (Heidelberg Engineering, Germany) was used for all IVCM imaging in this study. Briefly, the confocal microscopist applanated the IVCM onto the cornea anaesthetised with 0.5% proparacaine eye drops (Aurocaine, Aurolab, Madurai, India), and a new IVCM sterile disposable cap was used for each patient. After manually focusing, a series of volume scans was recorded at the centre and margins of the ulcer (12, 3, 6 and 9 o'clock positions), with overlapping volume scans taken from surface epithelium to the deepest region of the ulcer that could be imaged. Each volume scan had a z-stack of 40 sections, each section measuring 400 µm×400 µm (384×384 pixels) with optical thickness of 2 µm.

IVCM image sets had patient-identifying data and microbiological diagnoses removed and were allocated a random study number. All image sets were shuffled into a random order before measurements were performed.

Branch angle measurement

Every branching hyphae present in each IVCM image within each section of each volume scan was measured by a single IVCM grader (JDC), who was masked to the microbiological diagnosis. Volume scan z-stacks were imported into ImageJ V.1 analysis software (National Institutes of Health, Bethesda, Maryland, USA) as best resolution jpeg files. For each participant, all measurable branch angles present in all section images were measured; all 40 sections in every volume scan were screened and measured for each participant. Branch angles were measured using the angle tool in ImageJ V.1. Presence or absence of dichotomous branching in all sections was also assessed. Data were recorded in Microsoft Excel for Mac 2011 (V.14.6.5).

Microbiological culture

Immediately after IVCM imaging, corneal scrapes were taken for microbiological culture using a sterile kimura spatula after application of 0.5% proparacaine eye drops. The confocal microscopist performing the scan and the IVCM grader were masked to the microbiological culture result. Also, the microbiologists were masked to the IVCM result. Corneal scrapings obtained from the base and leading edge of the ulcer were placed directly on to blood agar and potato dextrose agar plates as well as a glass slide (for 10% potassium hydroxide staining to aid visualisation of fungal hyphae). Standard microbiological procedures were followed to identify organisms, as previously reported.[12] Fungal culture positivity was reported if any of the following criteria were found: (a) growth of the same fungal species on ≥2 solid media or (b) semiconfluent growth at the site of inoculation in one solid medium. Fungal species were identified by morphology of the fungal colony, and lactophenol cotton blue stained hyphae and spores.[13] Although the ocular microbiology laboratory were able to identify some but not all *Fusarium* subspecies, for this study we recorded all Fusarial growth as '*Fusarium* spp', as per the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) guidance, which recommends molecular typing above morphological features for accurate *Fusarium* subspecies classification.[14]

Statistical analysis

All analyses were performed with Stata V.12.1 (StataCorp, College Station, Texas, USA). Difference in baseline demographic features between each group were compared using the Kruskal-Wallis test for non-normally distributed variables (age, symptom duration, ulcer size) and χ^2 test for proportions (gender, prior drug use, presence of diabetes, presence of deep infiltrate). Branch angles were analysed as clustered data by patient, using a linear mixed-effects model (xtmixed) with restricted maximum likelihood estimation (reml); backwards stepwise regression was used to select predictor variables with likelihood ratio tests used for significance testing at each stage. A threshold value of p=0.10 was used for exclusion of variables, leaving fungal species, presence of a deep ulcer and prior antifungal use in the multivariate regression model, with adjustment for age and gender.

RESULTS

During February 2012–February 2013, 106 patients with keratitis were recruited, who were culture positive for *Fusarium* or *Aspergillus* spp. These patients form part of a larger cohort of 239 patients with microbial keratitis for whom we calculated the sensitivity (85.7%; 95% CI 82.2% to 88.6%) and specificity (81.4%; 95% CI 76.0% to 85.9%) of IVCM in the diagnosis of fungal keratitis compared with culture and light microscopy, full results published elsewhere.[15] Eight patients were excluded from the IVCM analysis (5 *Fusarium* spp, 2 *Aspergillus flavus*, 1 *A. fumigatus*) due to the absence of any measurable branching hyphae in the IVCM images. In the remaining 98 participants, 68 were culture-positive for *Fusarium* spp, 24 for *A. flavus*, 4 for *A. fumigatus* and 2 for *Aspergillus terreus*. A median of 11 IVCM volume scans were obtained for each patient (range 3–28). A total of 1254 images were assessed for presence of any branching angle. Branch angles were detected and measured in 627 images in total (median 5 images measured per patient, range 1–19).

There was no significant difference between the *Fusarium* and *Aspergillus* spp groups in gender, ulcer stromal infiltrate size, presence/absence of diabetes mellitus or prior use of topical

antifungals or steroid (see table 1). Participants who were culture positive for *Aspergillus* spp were older compared with those with *Fusarium* spp (median age 54 vs 45 years), and had a longer median symptom duration (7 vs 5 days, see table 1). A higher proportion of patients with *Aspergillus*-positive keratitis presented with deep ulcers involving the posterior third of the cornea compared with the *Fusarium*-positive group (80% vs 54%, see table 1). Data on topical medication used prior to presentation were available for 87 patients, of whom 45% (n=39) had used an antifungal beforehand, specifically 21% (n=18) had used a polyene antifungal (eg, natamycin), 6% (n=5) had used an azole antifungal (eg, voriconazole) and 18% (n=16) had used both drugs. Also, 10% (n=9) had used a topical steroid, 2% had used a steroid in conjunction with an antifungal. A small number of patients had a coexisting diagnosis of diabetes mellitus (n=11); evidence was very weak (p=0.068) of an association between diabetes and fungal species.

Branching angle of *Fusarium* spp versus *Aspergillus* spp

The mean branching angle for filaments of *Fusarium* spp was 59.7° (95% CI 57.7° to 61.8°) and for *Aspergillus* spp was 63.3° (95% CI 60.8° to 65.8°). The small difference in branching angle between the two species did not reach significance at the 5% level in univariate analysis, but became significant when adjusting for age and gender (table 2). Figure 1 shows IVCM images from culture-positive *Fusarium* and *Aspergillus* spp ulcers, highlighting the similarity in branching structure. Branching angle from all species together were not significantly affected by symptom duration, presence of diabetes mellitus, ulcer size or prior steroid or antifungal use (table 2). Multivariate analysis including depth of ulcer, adjusting for age and gender, showed that the branching angle for *Aspergillus* spp was 4.8° greater than for *Fusarium* spp (95% CI 1.0° to 8.5°, p=0.012) and that deeper ulcers had a branching angle of 4.0° smaller than all others (95% CI −0.3° to −7.7°, p=0.034; see table 3).

Among *Fusarium* spp ulcers, deep ulcers had a smaller branch angle by 4.3° (57.8° (95% CI 55.0° to 60.6°) vs 62.1° (95% CI 59.2° to 64.9°) in superficial ulcers; p=0.037). Deep *Aspergillus* spp ulcers also had smaller branch angles but the difference did not reach statistical significance (62.7° (95% CI 59.6° to 65.8°) vs 65.1° (95% CI 61.0° to 69.2°), p=0.444). Prior antifungal use also caused a significant reduction in branch angle among *Fusarium* spp, but only by 5.9° (56.6° (95% CI 52.6° to 60.6°) vs 62.5° (95% CI 60.2° to 64.9°); p=0.037). However, there was no significant effect of antifungal use on branch angle in *Aspergillus* spp (63.4° (95% CI 60.2° to 66.6°) vs no antifungal use 63.2° (95% CI 59.3° to 67.1°); p=0.99).

Only five corneal ulcers had a mean fungal branching angle ≤45°, of these four were culture positive for *Fusarium* spp and one for *A. flavus*. The remaining ulcers had mean branching angles of >45° and were positive for *Fusarium* spp (n=64) or *Aspergillus* spp (n=29). Two ulcers had at least one branch angle measured as 90° and both were caused by *Fusarium* spp.

Table 1 Demographic data and clinical features of study participants

	Fusarium spp keratitis (n=68)	*Aspergillus* spp keratitis (n=30)	p Value
Median age, years (range)	45 (20–70)	54 (30–79)	0.016
Male gender, n (%)	50 (73.5)	21 (70.0)	0.719
Symptom duration, median no. of days (range)	5 (1–90)	7 (2–30)	0.003
Diabetes mellitus present, n (%)	5 (7)	6 (20)	0.068
Prior use of any antifungal, n (%)*	24 (41.4)	15 (51.7)	0.360
Prior topical steroid/ciclosporin use, n (%)*	6 (10.3)	3 (10.3)	1.000
Stromal infiltrate longest diameter, mm (range)	5.0 (3.0–10.0)	5.0 (3.0–8.3)	0.260
Presence of deep infiltrate in posterior third of cornea (assessed at slit lamp), n (%)	37 (54.4)	24 (80.0)	0.016

*For prior medication usage, n=58 for *Fusarium* group, n=29 for *Aspergillus* group (data not available for n=11).

Table 2 Effect of fungal species, sociodemographic and clinical features on fungal branching angle (analyses adjusted for age and gender)

	Change in branch angle (°)	95% CI (°)	p Value
Aspergillus spp (compared with *Fusarium* spp)	4.2	0.5 to 7.9	0.025
Presence of deep infiltrate in posterior third of cornea	−3.4	−7.2 to 0.3	0.075
Symptom duration (days)	−0.1	−0.3 to 0.1	0.553
Diabetes mellitus	1.2	−4.0 to 6.4	0.654
Prior steroid/ciclosporin use	1.2	−4.1 to 6.6	0.650
Stromal infiltrate diameter (mm)	−0.9	−1.9 to 0.2	0.113
Prior antifungal use	−3.1	−6.6 to 0.3	0.076

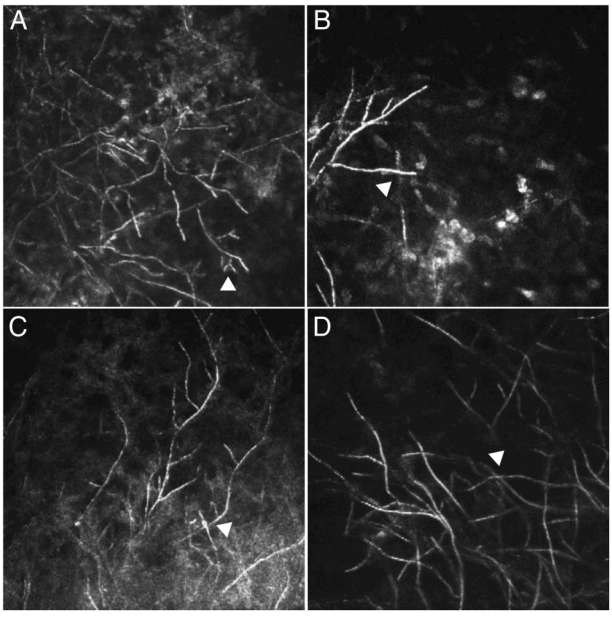

Figure 1 In vivo confocal microscopy (IVCM) images of keratitis caused by (A) *Aspergillus flavus* and (B), (C) and (D) *Fusarium* spp (image size 400×400 microns). All branching angles detected in all images measured <78°. Arrowhead in (A) shows dichotomous branching and in (B) shows two fungal filaments overlapping giving the false impression of a 90° branch angle. (C) and (D) show IVCM images representative of the keratitis seen in this study with arrowheads showing overlapping filaments.

Table 3 Results of multivariate regression model showing effect of fungal species and ulcer depth on branch angle (adjusted for age and gender)

	Change in branch angle (°)	95% CI (°)	p Value
Aspergillus spp (compared with *Fusarium* spp)	4.8	1.0 to 8.5	0.012
Presence of deep infiltrate in posterior third of cornea	−4.0	−7.7 to −0.3	0.034

There was a small variation in the mean branching angle within the *Aspergillus* spp, with the smallest being *A. fumigatus* at 59.1° (95% CI 53.5° to 64.6°), then *A. flavus* at 63.4° (95% CI 60.4° to 66.4°), and finally *A. terreus* at 68.9° (95% CI 58.4° to 79.5°).

Other morphological features

Presence or absence of dichotomous branching was assessed in 92 patients (total 203 images, median 2 images per patient, range 1–10); image sets from six patients were excluded as the fungal filaments in the images were inadequate to detect this feature. Dichotomous branching was infrequently seen, detected in only seven ulcers in the *Aspergillus* spp group, compared with four in the *Fusarium* spp group (25.9% vs 6.1%).

All *Fusarium* spp ulcer images were assessed for presence of any adventitious sporulation, but this morphological feature was not detected in any of these images.

DISCUSSION

IVCM allows for rapid identification of fungi directly within the patient's cornea. Previous case reports have suggested that the fungal branching angle detected in IVCM imaging of keratitis can be used to differentiate between fungal species, with a 90° angle reported in *Fusarium* spp[8] and 45° in *Aspergillus* spp.[6–8] There is increasing evidence that keratitis caused by *Aspergillus* spp may have worse clinical outcomes, higher risk of serious complications such as corneal perforation and respond less well to natamycin therapy compared with *Fusarium* spp. Therefore, it is advantageous to be able to distinguish between the two species early on in the clinical course.[3 16]

In this large prospective study, we found that *Fusarium* and *Aspergillus* spp had very similar branching angles as measured in IVCM images from patients with microbial keratitis; the difference between the two species was very small (~4°) and became statistically significant when adjusting for age and gender. Our findings are consistent with histopathological studies, which have shown on morphological appearances alone, misdiagnosis of fungal species can occur since several fungi produce hyphae with similar morphological appearance to *Aspergillus* or *Fusarium* spp, and that in fact these two species themselves can be indistinguishable.[9] Previous studies have compared culture results with histopathological diagnosis in tissue biopsies and have found discordant results in up to 35% of samples, particularly for *Aspergillus* and *Fusarium* spp infections. Lee *et al*[17] found that 17% (9/53) of tissue biopsy specimens were diagnosed as *Aspergillus* spp based on histopathological appearance of acute-angle branching hyphae, but were actually culture positive for a variety of non-*Aspergillus* organisms including *Fusarium* spp, *Scedosporium* spp, *Pseudallescheria* spp, *Phialophora verrucosa* and *Trhichophyton* spp. Schofield *et al*[18] found that in 35% (8/23) tissue biopsies from infected burn wounds that were classified by histopathology as having hyphae consistent with *Aspergillus* spp, the causative organism isolated in culture was actually *Fusarium* spp, *Trichosporon* spp, *Curvularia* spp or *Candida* spp. A further study reported misclassification of biopsy specimens in 21% of cases (n=10/47), including three cases reported as *Aspergillus* spp on histopathology but culture positive for *Fusarium* spp, *Scedosporium* spp or *Rhizopus* spp.[19] Since such misclassifications may have an impact on starting correct antimicrobial therapy, recent guidelines recommend using both histopathology and culture results together to make the diagnosis of invasive aspergillosis and fusariosis.[14 20]

Fungal filament appearance, branching angle and growth can be affected by several factors, including topography of the host tissue,[21] local availability of oxygen or nutrients (eg, glucose), tendency to grow away from neighbouring hyphae (known as negative autotropism) as well as health of the fungal cell wall and cell membrane.[22] Both polyene and the azole antifungals act on ergosterol, a major component of the fungal cell membrane, to disrupt hyphal growth.[23 24] Both *Fusarium* and *Aspergillus* spp had smaller branch angles when treated with antifungal prior to presentation or in deep ulcers but only reaching statistical significance for *Fusarium* spp. However, the magnitude of this effect (ie, <6° at most) was very small and therefore may not have masked any true difference in branch angle between the two species. Further studies are therefore required to more fully explore the impact of different antifungals on fungal morphology.

In addition to branch angle, other characteristic morphological features have been noted in culture and/or histopathology for some keratitis-causing fungi. *Fusarium*, *Acremonium* and *Paecilomyces* spp can sometimes develop spores within the infected tissue, known as adventitious sporulation, and this has also been detected in keratitis.[10] In our series of *Fusarium*-positive ulcers, we were unable to detect adventitial sporulation. This may be due to the size of the fungal spores reaching the limits of resolution of the laser scanning HRT confocal microscope (ie, 2 microns laterally).[13 25] *Aspergillus* spp have been described to have dichotomous branching, where the tip of the distal fungal hyphae splits into two branches.[9] Although we did detect this phenomenon in a higher proportion of ulcers positive for *Aspergillus* spp than in the *Fusarium* spp, further studies with a larger sample of *Aspergillus* keratitis are required to confirm this finding.

This study had several limitations. The IVCM images obtained in this study were two-dimensional and therefore the three-dimensional (3D) structure of the fungal mycelium growing within the entire cornea could not fully be appreciated. Advances in IVCM imaging may allow 3D reconstruction of HRT3/Rostock Corneal Module images of the entire cornea in the future,[25] thus enabling research into the relationship between hyphal growth, branching and tissue topography. We found that the majority of ulcers in the study were culture positive for *Fusarium* spp. This pattern of increased incidence of keratitis due to *Fusarium* spp in this geographical region has been previously reported.[2] Participants with culture-positive *Aspergillus* spp ulcers more frequently had a longer symptom duration and deeper corneal involvement at presentation, but all of these parameters still only impacted on branch angle by a few degrees.

In summary, we have found very little difference between the hyphal branching angle in IVCM images taken from culture-positive *Fusarium* and *Aspergillus* spp ulcers. Although IVCM remains a valuable tool to detect fungal filaments in corneal ulcers, it cannot be used to distinguish *Fusarium* spp from *Aspergillus* spp and culture remains essential to determine fungal species.

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Competing interests None declared.

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Title: In vivo confocal microscopy appearance of *Fusarium* and *Aspergillus* species in fungal keratitis

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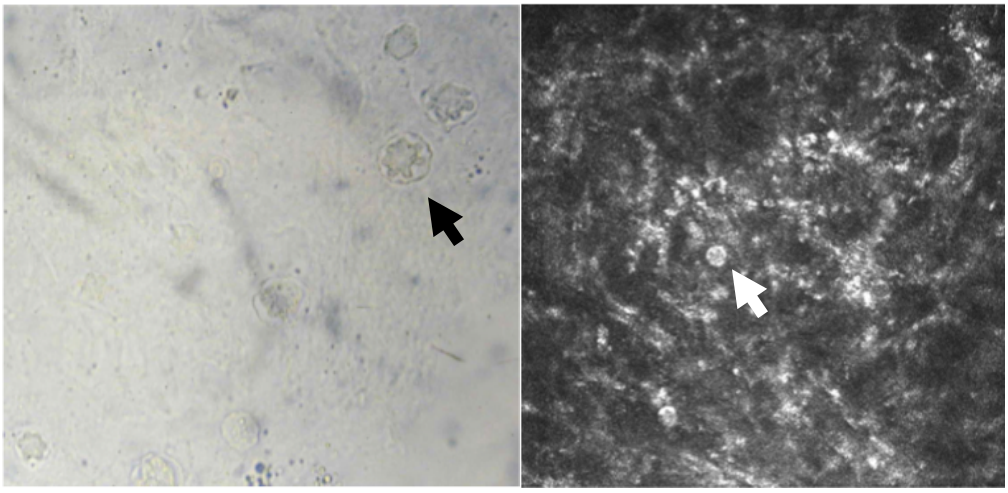
Chapter 6

In vivo confocal microscopy cellular features of bacterial, fungal and *Acanthamoeba* keratitis



Acanthamoeba double-walled cyst seen with light microscopy (image on left, black arrow) and in HRT3 *in vivo* confocal microscopy image from keratitis patient at Aravind Eye Hospital (image on right, white arrow)

6.1 Preamble

The manuscript in Chapter 6 describes the morphological differences within the host cornea (i.e. cellular and structural features) in bacterial, fungal and *Acanthamoeba* keratitis in IVCM images from the cohort study population at presentation.

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Principal Supervisor	Matthew Burton
Thesis Title	Studies in the Diagnosis and Pathophysiology of Severe Microbial Keratitis

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For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I designed the study with assistance from co-authors Dr. M. Burton, Dr. Prajna, & Dr. Lalitha). I recruited study participants (with help from Ms. Elakkiya & Dr. Shah), performed ulcer slit lamp examinations and IVCM (along with co-authors Dr. P. Srikanthi & Dr. S. Lanjewar). I performed all of the IVCM image analysis. I did the statistical analysis with guidance from Dr. Macleod. I wrote the manuscript, and included edits as suggested by co-authors.

Student Signature: [redacted] **Date: 8/8/18**
Supervisor Signature: [redacted] **Date: 8/8/18**

In Vivo Confocal Microscopy Cellular Features of Host and Organism in Bacterial, Fungal, and *Acanthamoeba* Keratitis

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• 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/).)

IN VIVO CONFOCAL MICROSCOPY (IVCM) HAS BEEN found to be a useful aid in the detection of organisms such as *Acanthamoeba* and fungi in human microbial keratitis (MK).[1] Laser-scanning HRT3 IVCM enables higher-resolution imaging compared to white light IVCM (eg, Confoscan), thus allowing better visualization of cellular changes during healing in the cornea.[2] Normal keratocytes have a specific appearance in IVCM images, with bright ovoid nuclei and barely visible cellular processes.[2] After injury, keratocytes differentiate into fibroblasts and then myofibroblasts,[3] and reduce their production of molecules that contribute to cellular transparency (eg, corneal crystallins), allowing greater visibility of their cellular processes with IVCM.[4,5] It has also been postulated that cellular changes associated with apoptosis may be visualized with IVCM, with absence of visible nuclei within cells and a granular appearance of intracellular contents.[2] Presence of "spindle-like" opacities within cells in IVCM images of the cornea may represent intracellular actin or microtubules associated with myofibroblasts, as observed in immunohistochemical studies of these cell types.[6] Additional cellular changes that have been noted using IVCM in MK are an increase in dendritiform cells (DFCs) (which become enlarged with long processes compared to their appearance in the normal cornea)[7] and an influx of inflammatory cells.[8] After corneal abrasion in a mouse model, HRT3 IVCM live imaging has shown that the initial inflammatory cells entering the stroma after injury migrate preferentially along keratocyte cellular processes and these processes are interconnected in a stellate, honeycomb-like pattern.[9]

Although many studies have looked at the diagnostic accuracy of IVCM for identifying a pathogen,[1,10–12] few have

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looked at the cellular changes in the cornea observed using IVCM in human MK.[7,8,13] Since the molecular changes that occur in human MK caused by different organisms are subtly different,[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," "double-walled cyst," or "signet ring" appearance of *Acanthamoeba* cysts[15,16]; spore-like structures along fungal filaments,[17] or fine-beaded filamentous appearance of *Nocardia* sp.[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).[1] 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 >80% corneal thinning as assessed by slit-lamp examination (since applanation 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.[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 applanated 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.[1] 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.[1]

• **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 broad, bright interconnected cellular processes 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

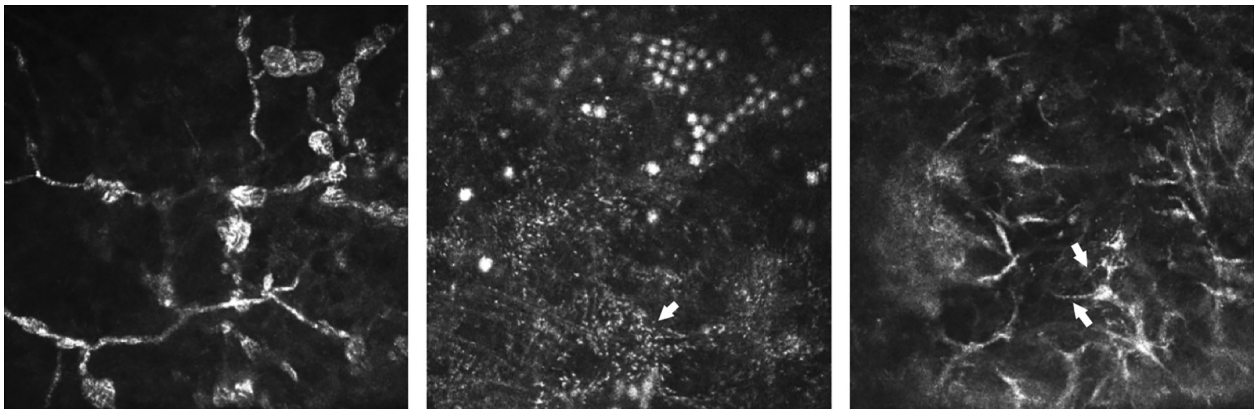

FIGURE 1. In vivo confocal microscopy (IVCM) images showing: (Left) spore-like structures along fungal filaments; (Middle) *Acanthamoeba* cysts forming lines and clusters (with presence of granules within the stellate interconnected cellular processes shown by arrow); and (Right) fine beaded filamentous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

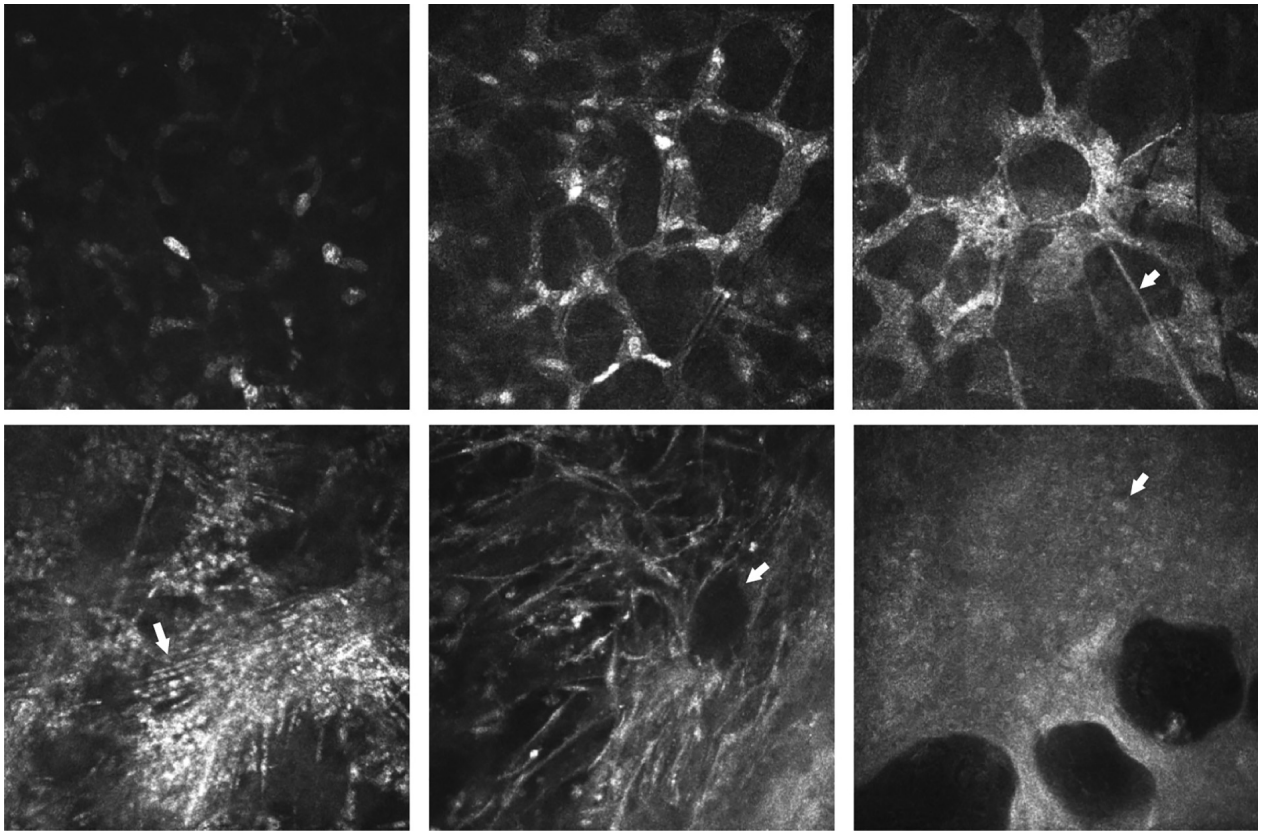

FIGURE 2. In vivo confocal microscopy images. (Top left) Normal keratocyte-like morphology (bright ovoid nuclei, barely visible cellular processes); (Top middle) "stellate intercellular connectivity with nuclei visible" (bright ovoid nuclei and broad bright cellular processes interconnected in a honeycomb 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).

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.

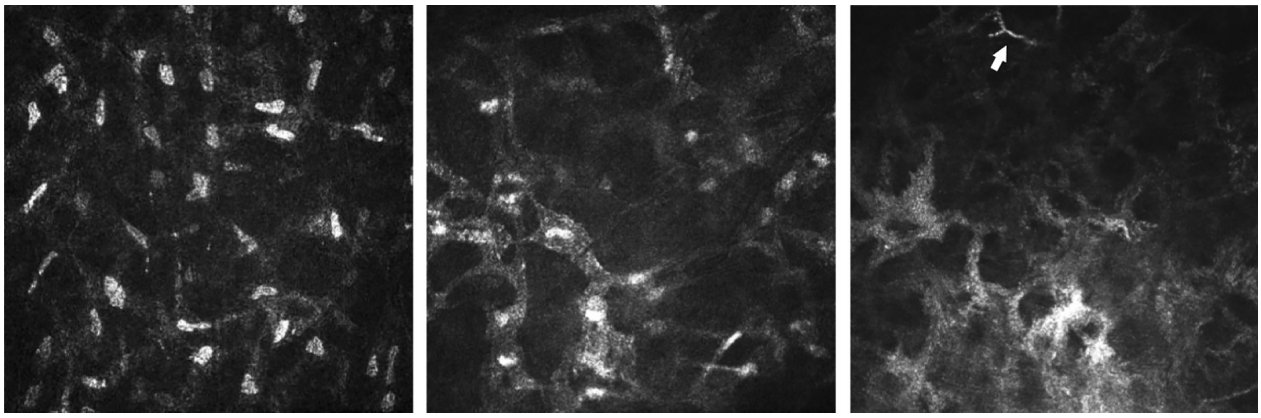

FIGURE 3. Additional in vivo confocal microscopy images show the normal keratocyte-like morphology in the anterior stroma (Left), stellate interconnected cellular processes with nuclei (Middle), and stellate interconnected without nuclei (Right; arrow highlights fungal filament).

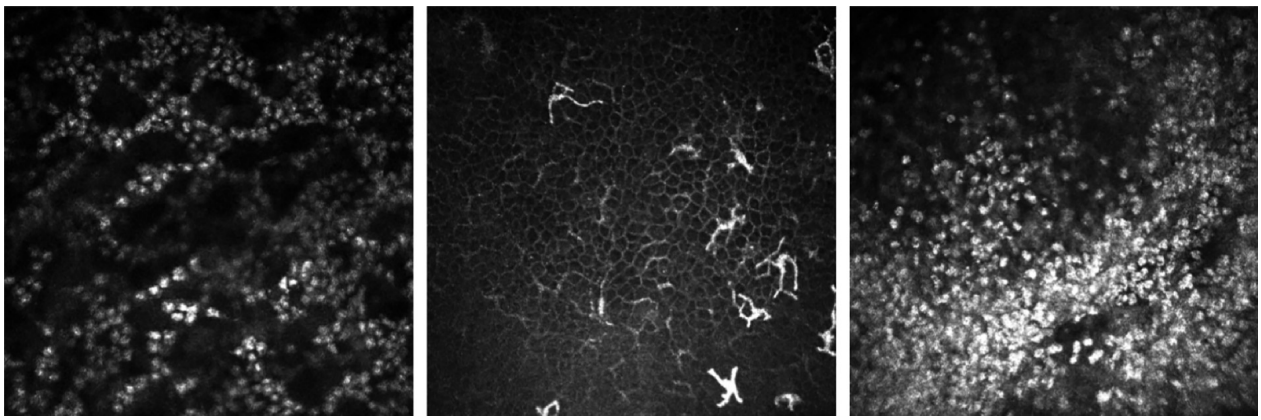

FIGURE 4. Appearance of inflammatory cell infiltrate in in vivo confocal microscopy images of microbial keratitis: (Left) inflammatory cells in a honeycomb distribution; (Middle) "dendritiform cells" in the basal epithelial layer; (Right) inflammatory cell infiltrate in a nonspecific distribution in the anterior stroma.

• **STATISTICAL METHODS:** 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.[20] Baseline demographic features and IVCM features were compared between BK, AK, FK, and microbiologically negative groups using χ^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.[1] 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 microbiologically 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 those 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).

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

	N	%
Fungi (n = 183)		
Culture-positive (n = 144):		
Fusarium sp.	73	33.5%
Aspergillus sp.	33	15.1%
Curvularia sp.	5	2.3%
Exserohilum sp.	4	1.8%
Lasiodiplodia sp.	2	0.9%
Cylindrocarpon sp.	1	0.4%
Bipolaris sp.	1	0.4%
Unidentified hyaline fungi	14	6.4%
Unidentified dematiaceous fungi	11	5.0%
Culture-negative but light microscopy–positive for fungi	30	13.8%
Culture-negative but IVCM-positive for fungi	9	4.1%
Acanthamoeba (n = 18)		
Culture-positive	17	7.8%
Culture-negative but IVCM-positive for *Acanthamoeba*	1	0.5%
Bacteria (n = 17)		
Culture-positive (n = 17):		
Streptococcus pneumoniae	9	4.1%
Nocardia sp.	3	1.4%
Pseudomonas aeruginosa	2	0.9%
Aeromonas sp.	1	0.4%
Streptococcus viridans	1	0.4%
Staphylococcus epidermidis	1	0.4%
Total	218	100%

IVCM = in vivo confocal microscopy.

• **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 2. Baseline Characteristics of Study Participants

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

[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 χ^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

Corneal Location	IVCM Features	Fungal Keratitis (N = 183)	Acanthamoeba Keratitis (N = 18)	Bacterial Keratitis (N = 17)	*P* Value[a]
Anterior	Normal keratocyte-like morphology	141 (77%)	7 (39%)	14 (82%)	.001
	Stellate cellular processes with nuclei	119 (65%)	6 (33%)	11 (65%)	.029
	Stellate cellular processes no nuclei	58 (32%)	10 (56%)	3 (18%)	.047
	Spindles	132 (72%)	14 (78%)	13 (76%)	.826
	Granules	108 (59%)	13 (72%)	11 (65%)	.514
	Epithelial bullae	18 (10%)	0 (0%)	7 (41%)	<.001
	Stromal bullae	19 (10%)	1 (6%)	8 (47%)	<.001
	Inflammatory cells (honeycomb)	90 (49%)	1 (6%)	6 (35%)	.001
	Inflammatory cells (nonspecific)	42 (23%)	3 (17%)	6 (35%)	.403
	Basal DFCs	97 (53%)	7 (39%)	14 (82%)	.027
	Stromal DFCs	19 (10%)	5 (28%)	4 (23%)	.043
	Scar	19 (10%)	4 (22%)	3 (18%)	.251
	Fungal spore-like structures	6 (3%)	0 (0%)	0 (0%)	-
Corneal Location	IVCM Features	Fungal Keratitis (N = 46)	Acanthamoeba Keratitis (N = 7)	Bacterial Keratitis (N = 4)	*P* Value[a]
Posterior	Normal keratocyte-like morphology	31 (67%)	4 (57%)	4 (100%)	.320
	Stellate cellular processes with nuclei	35 (76%)	2 (29%)	3 (75%)	.037
	Stellate cellular processes no nuclei	10 (22%)	5 (71%)	1 (25%)	.024
	Spindles	30 (65%)	6 (86%)	2 (50%)	.430
	Granules	22 (48%)	6 (86%)	2 (50%)	.173
	Inflammatory cells (honeycomb)	11 (24%)	1 (14%)	0 (0%)	.476
	Inflammatory cells (nonspecific)	8 (17%)	0 (0%)	2 (50%)	.111
	Stromal DFCs	2 (4%)	0 (0%)	0 (0%)	.780
	Scar	0 (0%)	1 (14%)	0 (0%)	.026
	Fungal spore-like structures	2 (4%)	0 (0%)	0 (0%)	-

DFCs = dendritiform cells; IVCM = in vivo confocal microscopy.
[a]Statistical significance of difference between all 3 groups assessed using χ^2 test.

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 4. Univariable and Multivariable Odds Ratios for In Vivo Confocal Microscopy Features Associated With Bacterial, *Acanthamoeba*, and Fungal Keratitis

Pathogen	Corneal Location	Variable	Univariable OR (95% CI)	*P* Value	Multivariable OR (95% CI)	*P* Value
FK vs all others	Anterior	Stromal bullae	0.31 (0.11-0.82)	.018	0.31 (0.11-0.82)	.018
		Honeycomb inflammatory cell distribution	2.74 (1.01-7.40)	.047	-	-
		Honeycomb inflammatory cell distribution with no stromal bullae	3.31 (1.02-10.77)	.046		
		Honeycomb inflammatory cell distribution with stromal bullae present	0.47 (0.15-1.45)	.189		
	Posterior	-	-	-	-	-
Aspergillus sp. vs *Fusarium* sp.	Anterior	Stellate cellular processes with nuclei	0.23 (0.09-0.61)	.003	0.24 (0.09-0.65)	.005
		Stromal dendritiform cells	11.17 (1.65-75.44)	.013	11.05 (1.49-82.13)	.019
		Normal keratocytes	0.32 (0.11-0.93)	.036	-	-
		Granules	0.41 (0.16-1.02)	.055	-	-
		Anterior broken hyphae	2.46 (0.89-6.83)	.084	-	-
		Spindles	0.42 (0.15-1.17)	.097	-	-
	Posterior	-	-		-	-
AK vs all others	Anterior	Normal keratocyte-like morphology	0.21 (0.06-0.79)	.022	0.21 (0.06-0.79)	.022
	Posterior	Stellate cellular processes with nuclei	0.08 (0.01-1.13)	.062	0.03 (<0.01-1.09)	.056
		Granules	25.01 (0.73-855.51)	.075	49.57 (0.94-2604.52)	.053
Steroid use vs all others	Anterior	*Acanthamoeba* cysts in cluster formation	9.98 (1.02-97.96)	.048	9.98 (1.02-97.96)	.048
		Acanthamoeba cysts in line formation	2.43 (0.36-16.48)	.363	-	-
BK vs all others	Anterior	Stromal bullae	9.99 (3.11-32.06)	<.001	9.99 (3.11-32.06)	<.001
		Epithelial bullae	5.72 (1.73-18.94)	.004	-	-
		Basal epithelial dendritiform cells	3.74 (1.00-13.91)	.049	-	-
		Stromal dendritiform cells	3.51 (0.88-14.09)	.076	-	-
	Posterior	-	-	-	-	-

AK = *Acanthamoeba* keratitis; BK = bacterial keratitis; CI = confidence interval; FK = fungal keratitis; OR = odds ratio.

FK sub-group analysis for *Aspergillus* sp. vs *Fusarium* sp. also shown. All analyses were adjusted for age, sex, ulcer size, and symptom duration.

• **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, $P = .049$) 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 within the IVCM images of 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

through action of cell adhesion molecules.[9] 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 structures 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 *Curvularia* 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 prognostic 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 clusters, 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 series 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*e 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 associates 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.[7] 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 steroid 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 elongated, 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 endothelial 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[37] and excessive stimulation of host cells (eg, neutrophils) to release matrix metalloproteinases that can also destroy host tissue.[38] Control of this damage through use of topical steroid 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.[39] IVCM may be a useful tool for monitoring the effect of any treatment regime on the stromal necrotic response.

Nocardia sp. have been documented to appear as thin beaded filamentous structures in IVCM images[18,19] and are therefore one of the few bacterial causes of keratitis that can be visualized with this imaging modality.[15] *Nocardia* sp. filaments are smaller in diameter than *Aspergillus* or *Fusarium* fungal hyphae (up to 1 μm compared to 3-6 μm for filamentous fungi).[13,40] We only observed thin beaded filaments in the IVCM images of 1 out of the 3 *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 of MK to make the diagnosis of *Nocardia* keratitis.

A limitation of our study is that we were able to enroll only a small number of bacterial and *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 *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.

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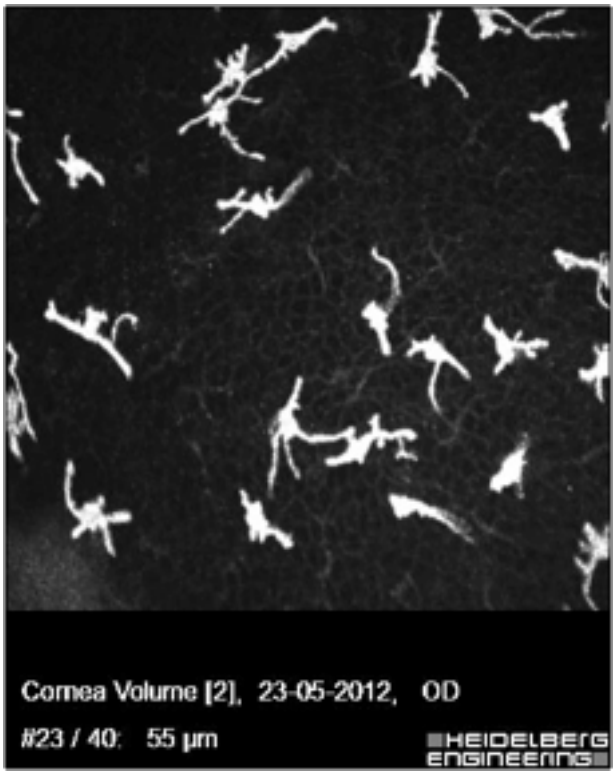
Chapter 7

Cellular morphological changes associated with clinical outcome in bacterial and fungal keratitis as detected with laser scanning *in vivo* confocal microscopy



Dendritiform cells observed in the corneal basal epithelial layer of a study patient with bacterial keratitis (culture-positive for *Streptococcus pneumoniae*).

7.1 Preamble

The manuscript in Chapter 7 describes the morphological changes observed in serial IVCM images of microbial keratitis in the cohort study population over the course of the study time period.

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PLEASE NOT THAT A COVER SHEET MUST BE COMPLETED FOR EACH RESEARCH PAPER INCLUDED IN A THESIS.

SECTION A – Student Details

Student	Jaya Chidambaram
Principal Supervisor	Matthew Burton
Thesis Title	Studies in the Diagnosis and Pathophysiology of Severe Microbial Keratitis

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?			
When was the work published?			
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion			
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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	Scientific Reports
Please list the paper's authors in the intended authorship order:	JD Chidambaram, NV Prajna, P Srikanthi, S Lanjewar, M Shah, S Elakkiya, P Lalitha, D Macleod, MJ Burton
Stage of publication	Not yet submitted

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I designed the study with assistance from co-authors Dr. M. Burton, Dr. Prajna, & Dr. Lalitha). I recruited study participants (with help from Ms. Elakkiya & Dr. Shah), performed ulcer slit lamp examinations and IVCM (along with co-authors Dr. P. Srikanthi & Dr. S. Lanjewar). I performed all of the IVCM image analysis. I did the statistical analysis with guidance from Dr. Macleod. I wrote the manuscript, and included edits as suggested by co-authors.

Student Signature: [redacted] **Date: 8/8/18**

Supervisor Signature: [redacted] **Date: 8/8/18**

Cellular morphological changes detected by laser scanning *in vivo* confocal microscopy associated with clinical outcome in fungal keratitis

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Running title: IVCM markers of outcome in microbial keratitis

Key words: in vivo confocal microscopy, keratitis, corneal ulcer, fungus, keratocyte

Abstract

Introduction: HRT3 *in vivo* confocal microscopy (IVCM) images may indicate outcome, but few studies have analysed this in fungal keratitis (FK).

Methods: Adults with FK (diameter ≥3mm) presenting to Aravind Eye Hospital, India from 2012-3 were enrolled prospectively. IVCM was performed at baseline, days 7, 14 and 21 post-enrolment (+/- 3 days where possible). Specific morphologies were identified in IVCM images by a grader masked to outcome/microbiology, and associations with final visit outcome (i.e. good: healed/improving, or poor: enlarged ulcer, perforation or transplant/glue) assessed using logistic regression.

Results: 143 FK participants were enrolled; 87 had good outcome, 56 had poor outcome. Poor outcomes were associated with stellate interconnected cellular processes with no visible nuclei (OR 2.28, 95% CI: 1.03-5.06, p=0.043) in baseline IVCM images, and fungal filaments (OR 6.48, 95% CI:2.50-16.78, p<0.001) or honeycomb distribution of inflammatory cells (OR 5.24, 95% CI: 1.44-19.06, p=0.012) in final visit images. Fungal filaments (OR 3.61, 95% CI:1.64-7.95, p=0.001), stromal dendritiform cells (OR 2.88, 95% CI:1.17-7.11, p=0.022), or stellate cellular processes with no visible nuclei (OR 2.09, 95% CI:1.14-3.82, p=0.017) were associated with poor outcome if not in baseline but present in final visit images.

Conclusions: IVCM can reveal morphological changes associated with outcome.

Introduction

After initiation of antimicrobial therapy for microbial keratitis (MK), it may be difficult to assess therapeutic response of some ulcers based upon clinical appearances alone.[1] *In vivo* confocal microscopy (IVCM) is a useful aid in detecting pathogens in the living cornea (e.g. fungal hyphae and *Acanthamoeba* cysts).[2] Specific cellular morphologies observed with IVCM, such as increase in the number of activated keratocytes have been previously postulated as early predictors of disease, e.g. graft rejection after penetrating keratoplasty.[3] Only a few studies have used IVCM to assess changes during the clinical course of MK.[1] Winchester *et al* used tandem-scanning IVCM in two patients with fungal keratitis (FK) and showed that *Aspergillus* hyphae broke into shorter fragments by day 4 post-natamycin therapy, and disappeared completely by day 12.[4] Shi *et al* used the confoscan IVCM to monitor 121 FK patients during antifungal therapy, and observed that fungal hyphae disappeared over time in parallel with reduction in the inflammatory cell infiltrate, however no quantitative data were presented on the association of specific IVCM cellular changes with outcome.[5] Heidelberg retinal tomography 3 IVCM with the Rostock Corneal Module (HRT3 IVCM) has a higher resolution than either the tandem-scanning or confoscan IVCM and therefore allows an improved view of cells within corneal tissue.[6] Cellular processes and nuclei of activated keratocytes can be observed in greater detail using the HRT3 IVCM[6], as well as other morphological appearances such as presence of round inflammatory cells and dendritiform cells.

To our knowledge, use of serial HRT3 IVCM to assess the cellular changes in MK has not been previously reported. Therefore, in this study we quantitatively assessed serial HRT3 IVCM images from a prospective cohort of severe bacterial and fungal keratitis patients in South India for morphological features that were associated with healing or worsening

clinical outcome. We chose to focus on moderate-to-severe keratitis in this study since these ulcers often present with an atypical clinical appearance and may be difficult to manage during their clinical course,[7] therefore we felt these ulcers would benefit the most from investigation of the cellular response with the use of IVCM.

Materials and 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 & Tropical Medicine, UK. All patients gave written informed consent prior to enrolment; illiterate participants gave informed consent with a witnessed thumbprint on the study consent form.

From February 2012 to February 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 $\geq$18 years, stromal infiltrate diameter $\geq$3mm, presence of overlying epithelial defect and signs of acute inflammation. Patients were excluded if the ulcer had a descemetocoele or >80% corneal thinning as assessed by slit lamp examination (since applanation for IVCM could not safely be done in these patients); clinical features or prior history of herpetic keratitis; Snellen visual acuity worse than 6/60 in the unaffected eye; *Acanthamoeba* keratitis diagnosed on culture and/or IVCM (these patients were followed up in a different study); or presence of mixed infection (i.e. culture-positive for bacteria and light microscopy and/or IVCM positive for fungus). At enrolment, data from a focused clinical history and slit lamp examination were recorded. The study follow-up visit schedule was as follows: days 7 (+/- 3 days), 14 (+/- 3 days) and 21 (+/- 3 days). Final visit assessment with IVCM imaging was included for patients unable to attend at 21 (+/- 3 days), but who

attended later up to a maximum of 37 days post-enrolment. Additional assessments were scheduled as clinically indicated. At each visit, the cornea consultant or cornea fellow examined every study participant and management followed the standard of care for microbial keratitis at Aravind Eye Hospital. Any participant with significant worsening of the ulcer despite maximal medical therapy (e.g. perforation or impending perforation, significant enlargement of the infiltrate) underwent surgical intervention (i.e. therapeutic penetrating keratoplasty; corneal glue for small perforations; intrastromal voriconazole for deep stromal abscess in fungal keratitis).

Clinical outcomes were assessed based on slit lamp examination of the cornea including measurements of the ulcer at each visit with grading as follows: 1) healed: no epithelial defect at final visit with presence of scar tissue; 2) improving: final visit infiltrate size less than enrolment size with or without epithelial defect still present; 3) worsening: final visit infiltrate size either the same as or larger than enrolment infiltrate size, if corneal perforation was noted (i.e. full-thickness hole seen in cornea at slit lamp, or iris plugging site of possible corneal perforation with flat anterior chamber and seidel-positivity), or if surgical intervention was performed. The "final visit" was defined as either the clinical assessment/IVCM imaging performed at the end of the study (i.e. up to 37 days post-enrolment) or the visit when the patient left the study due to surgical intervention.

IVCM Imaging

HRT3 IVCM imaging (Heidelberg Engineering, Germany) of the corneal ulcer was performed immediately prior to corneal scraping for microbiological tests, as described in detail elsewhere.[2,8] Proparacaine 0.5% eyedrop anaesthesia was used (Aurocaine, Aurolab, Madurai, India) and the RCM (Heidelberg Engineering, Germany) with 63x objective lens (Nikon,

Japan) was gently applanated to the corneal surface. Using the HRT3 IVCM (Heidelberg Engineering, Germany), volume scans were obtained in a systematic manner at the center of the ulcer and ulcer margins (12, 3, 6 and 9 o'clock positions). By manually refocusing the Rostock Corneal Module we obtained a series of volume scans covering consecutive 80 micron depths from epithelium to endothelium, where possible, in each of these cardinal locations at the ulcer margins and center. This imaging method has been previously used to systematically scan the cornea in prior publications.[2,9] IVCM imaging was repeated using the same methodology at each follow-up visit. After IVCM imaging, corneal scrapes were obtained from the leading margin and base of the ulcer for culture and light microscopy, using standard procedures described in detail elsewhere.[2]

IVCM Grading

IVCM volume-scan images were assigned a random identification number, had patient-identifying data removed, and were shuffled into a random order. A single experienced grader performed all image grading and was masked to the clinical features and microbiological diagnosis. Images from each visit were graded independently from all other visits. All data were recorded in a Microsoft Access database. The grading scheme included presence/absence of the following morphological features in any single IVCM image: fungal filaments (Figure 1a), corneal epithelial or stromal bullae (Figure 1b), dendritiform cells in either the basal epithelial layer or stroma and homogenous-acellular scar-like tissue (Figure 1c). Presence/absence of the following morphological features were also assessed: normal, quiescent keratocytes (i.e. bright ovoid nuclei with barely visible cellular processes; Figure 2a); stellate structures with bright interconnected broad cellular processes either with or without visible bright ovoid nuclei (Figure 2b and 2c respectively); inflammatory cells in either a honeycomb distribution (Figure 2d) or a non-specific distribution; linear "spindles";

or "granules" (i.e. 1-2μm diameter bright opacities within the nucleus or cellular processes; Figure 2c).

Figure 1: HRT3 *in vivo* confocal microscopy images at final visit showing: (a) fungal filaments in the corneal stroma (arrow shows activated keratocytes with granular intracellular contents), (b) stromal bullae shown by arrow, and (c) acellular scar tissue in lower half of image.

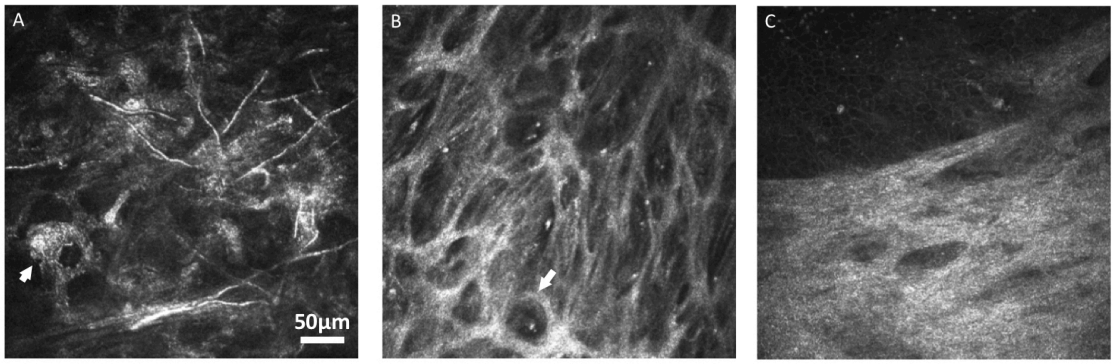
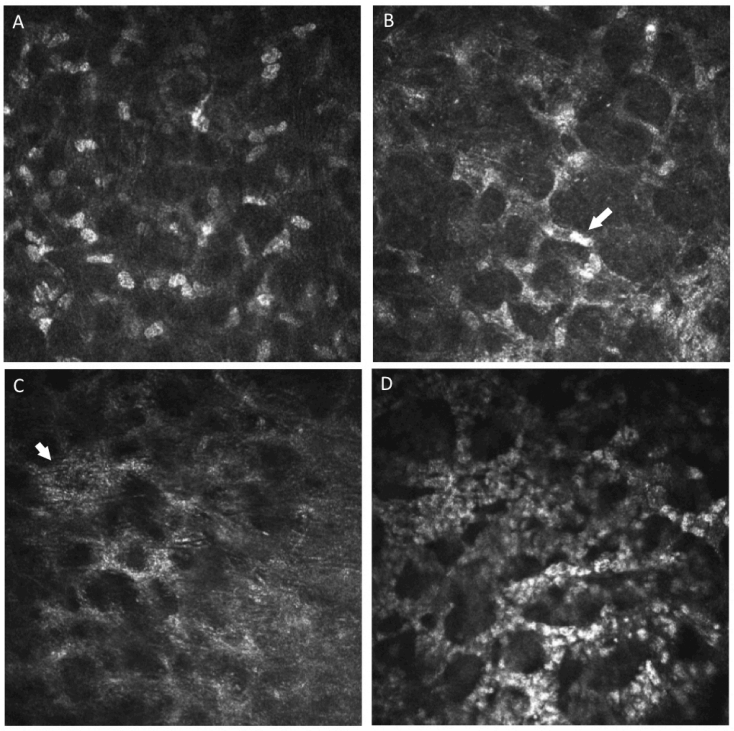


Figure 2: HRT3 *in vivo* confocal microscopy images of keratocytes in fungal keratitis showing: (a) normal keratocyte morphology (bright ovoid nuclei with barely visible cellular processes), (b) stellate interconnected cellular processes with bright ovoid nuclei (whon by arrow), (c) stellate interconnected cellular processes but no visible nuclei (arrow shows granular intracellular appearance), (d) inflammatory cells in a honeycomb distribution.



Statistical methods

All statistical analyses were performed in Stata 13.1 (StataCorp, Texas, USA). Logistic regression analysis was used to assess statistical significance of differences in morphological features in ulcers with poor outcome versus good outcome in the baseline visit IVCM images and then in the final visit images. Conditional logistic regression analysis was used to assess change in morphological features within each patient's images from baseline to the final visit, with poor versus good outcome as the dependent variable (i.e. although patients may have been followed up at different times for their final visit, this factor is taken into account as the conditional logistic regression analysis only analyses the change within each patient alone, not a comparison between patient groups). All regression analyses were adjusted for age, gender, baseline ulcer stromal infiltrate size (defined as the geometric mean of longest stromal infiltrate diameter and its perpendicular diameter) and time from symptom onset to clinic visit (for analyses that included the final visit data). Forwards stepwise selection was used to create the multivariable model (variables included if $p<0.1$ in univariable regression analyses and retained if $p<0.05$ in likelihood ratio test with full model with all selected variables included).

Results

Participants and microbiology

One-hundred and eighty-two participants with FK were initially enrolled into the study (FK diagnosed as follows: 143 culture-positive, 30 culture-negative/light microscopy positive, and 9 microbiologically-negative/IVCM-positive for fungi.) However, 39 patients were excluded from the study due to the following reasons: 7 lost to follow-up after the first visit; inability to perform IVCM at the final visit in 32 (due to prior corneal perforation in 21, excessive corneal thinning/descemetocele in 3, recent penetrating keratoplasty in 7, and 1 patient

unable to attend for IVCM imaging). In the 143 participants included in the study, a total of 1647 volume scans were performed (median 11 volume scans per patient, interquartile range 8-13), consisting of a total of 65,880 images that were assessed in this study.

Of the patients who reported use of any antimicrobial therapy prior to presentation (n=126), 23.8% had been using natamycin alone (n=30/126), 5.5% had been using an azole alone (e.g. voriconazole; n=7/126) and 27.8% had been using both natamycin and a topical azole in combination (n=35/126). After being seen in the cornea clinic, the 143 final study participants were commenced on the following intensive (hourly) antifungal eyedrops: 5% natamycin alone in 93, 5% natamycin and 1% voriconazole in 46, topical 1% itraconazole eyedrops in one, initial antibiotic (moxifloxacin) in one until fungal culture results noted at first follow-up visit then natamycin started; fortified cefazolin, levofloxacin and polymyxin B ointment in one patient whose ulcer had resolved by the time fungal results were available (i.e. culture-negative, equivocal IVCM images at presentation, but found to have few fungal filaments on IVCM imaging at a later visits once clinically resolving). An oral antifungal agent was given in 103 patients (e.g. itraconazole, ketoconazole, or fluconazole tablet) during the study. In ulcers that were found to be clinically worse or not improving at follow-up visits, antifungal treatment was changed as follows: topical voriconazole 1% was started in addition to natamycin in 17 patients, itraconazole 1% eye ointment was added in addition to natamycin in 18 patients, topical clotrimazole 1% eyedrops (in addition to natamycin alone or natamycin and topical voriconazole) was added in three patients.

With maximal medical therapy, by the final visit, 87 ulcers had a "good" outcome (i.e. 31 healed ulcers and 56 showing improvement by the final visit) and 56 had a "poor" outcome (i.e. 10 perforated, 46 worse or no improvement by the end of the study). Those with a

good outcome had slightly shorter symptom duration, smaller ulcer size with fewer deep ulcers at enrolment, compared to those with a poor outcome (Table 1). The main causative organism in those with good outcome was *Fusarium* sp. (n=43/87) and *Aspergillus* sp. (n=8/87).

Table 1: Baseline characteristics of 143 study participants by clinical outcome.

	Poor Outcome (n=56)	Good Outcome (n=87)	p-value[a]
Age, median years (IQR)	50 (37-60)	45 (55-35)	0.148
Gender, no. male, (%)	32 (57%)	62 (71%)	0.082
Symptom duration at presentation, median days, (IQR)	7 (4-15)	6 (4-10)	0.022
Days post-enrolment to final visit, median days (IQR)	16 (10-21)	21 (20-23)	<0.001
Baseline best-corrected visual acuity, logMAR (IQR)	1.8 (0.8-1.8)	1.0 (0.4-1.8)	0.058
Ulcer infiltrate size, median mm, (IQR)	5.0 (3.9-5.9)	3.7 (3.1-4.5)	<0.001
Posterior 1/3 of cornea affected, n (%)	39 (70%)	43 (49%)	0.017

[a]Statistical differences between groups assessed using either chi-squared test for proportions or kruskall wallis test for non-parametrically distributed continuous variables.

Of the 56 participants in the "poor" outcome group, 36 had an enlarged stromal infiltrate by the final visit, 10 had no change in the stromal infiltrate from the size at presentation, 10 had developed corneal perforation (of whom two had corneal glue), and a total of 16 patients underwent therapeutic penetrating keratoplasty. The commonest organisms cultured in poor outcome ulcers were *Fusarium* sp. (n=14/56), *Aspergillus* sp. (n=16/56, including 12

Aspergillus flavus) and dematiaceous fungi (n=5: *Cylindrocarpon* sp. 1, *Curvularia* sp. 1, *Exserohilum* sp. 2, *Lasiodiplodia* sp. 1). The 10 perforated ulcers were caused by *Fusarium* sp. (n=2), *Aspergillus flavus* (n=2), dematiaceous fungi (n=2: *Cylindrocarpon* sp. and *Exserohilum* sp.) or were culture-negative but light microscopy or IVCM-positive for fungi (n=4).

Cellular features in baseline and final visit IVCM images associated with good or poor outcome

The proportion of ulcers with presence of each of the cellular morphologies in baseline and final visit IVCM images are shown in Supplementary table 1. Logistic regression analysis of baseline images alone showed that poor outcome was significantly associated with the presence of a stellate appearance of interconnected cells without visible nuclei (multivariable OR 2.28, 95% CI: 1.03-5.06, p=0.043; Table 2), as shown in Figure 2c.

Multivariable analysis of IVCM images at the final visit showed poor outcome to be associated with the presence of inflammatory cells in a honeycomb distribution (OR 5.24, 95% CI: 1.44-19.06, p=0.012) or intact fungal hyphae (OR 6.48, 95% CI 2.50-16.78, p<0.001; Table 2). Good outcome was associated with presence of stromal scarring (OR 0.20, 95% CI: 0.08-0.54, p=0.001; Table 2).

Changes from baseline to final visit associated with good or poor outcome

Multivariable analysis identified three cellular features that were associated with worsening if they were absent at baseline but were present in final visit IVCM images: fungal filaments (OR 3.61, 95% CI: 1.64-7.95, p=0.001), stromal stellate cellular appearance with no visible nuclei (OR 2.09, 95% CI: 1.14-3.82, p=0.017) and dendritiform cells in the stroma (OR 2.88, 95% CI: 1.17-7.11, p=0.017; Table 2). Change in anterior stromal scar tissue over time, i.e.

none at baseline but scarring present at final visit, was the only feature associated with good outcome in this analysis (OR 0.22, 95% CI: 0.10-0.49, p<0.001, Table 2).

Table 2: Odds ratios (OR) from univariable and multivariable logistic regression models for poor outcome (compared to good outcome) and morphological features in IVCM images from (i) baseline only, (ii) final visit only, or (iii) features that change from being absent in baseline images to being present in final visit images. Models were adjusted for age, gender, baseline ulcer size and time from symptom onset to final visit (for models including final visit data).

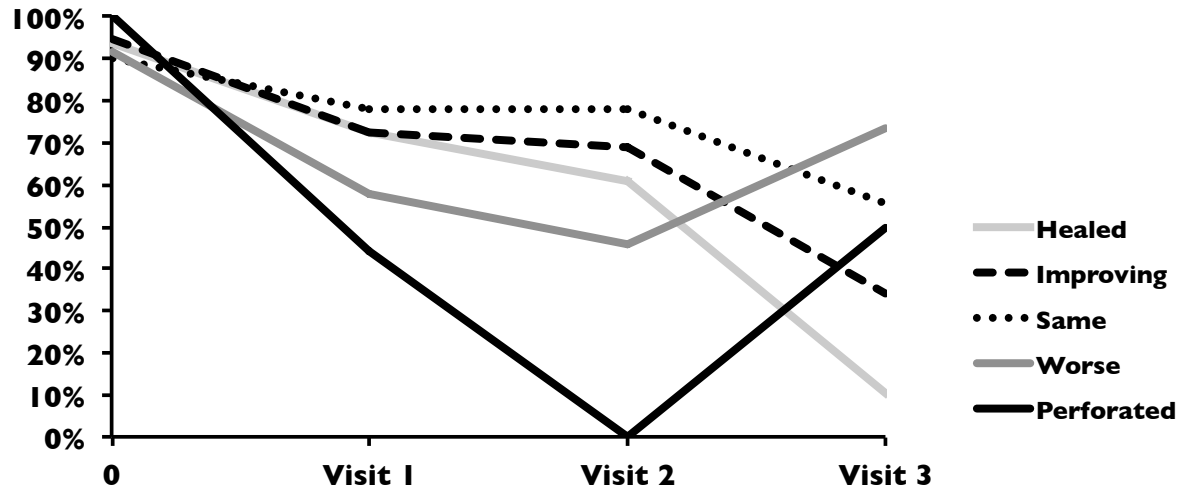
Morphological Feature	Univariable OR (95% CI)	p-value	Multivariable OR (95% CI)	p-value
(i) Baseline visit only				
Stellate cellular processes no nuclei	2.30 (1.04-5.08)	0. 039	2.28 (1.03-5.06)	0.043
Stromal dendritiform cells	2.99 (0.91-9.80)	0.070	2.93 (0.88-9.67)	0.077
(ii) Final visit only				
Fungal hyphae	7.25 (3.09-17.02)	<0.001	6.48 (2.50-16.78)	<0.001
Inflammatory cells (honeycomb)	6.42 (2.07-19.89)	<0.001	5.24 (1.44-19.06)	0.012
Scar	0.17 (0.07-0.40)	<0.001	0.20 (0.08-0.54)	0.001
Inflammatory cells (non-specific distribution)	2.50 (0.93-6.76)	0.070	-	-
(iii) Change from baseline to final visit*				
Fungal filaments	4.34 (2.07-9.10)	<0.001	3.61 (1.64-7.95)	0.001
Stromal dendritiform cells	2.06 (0.89-4.76)	0.090	2.88 (1.17-7.11)	0.022
Stellate cellular processes with no visible nuclei	1.61 (0.93-2.77)	0.088	2.09 (1.14-3.82)	0.017
Stromal scar	0.27 (0.13-0.54)	<0.001	0.22 (0.10-0.49)	<0.001
Inflammatory cells in honeycomb distribution	2.14 (1.28-3.89)	0.013	-	-

*Conditional logistic regression analysis performed comparing changes within each patient from baseline to final visit (i.e. not between patients).

Disappearance of fungal filaments in IVCM images over time and outcome

The proportion of patients with fungal filaments detected in IVCM images at each visit categorized by clinical outcome is shown in Figure 3. In ulcers that healed or had improved by the final visit, there was an initially steep and then consistent decline in the proportion of patients with visible fungal filaments in IVCM images at each sequential visit. However, ulcers that worsened or perforated showed reduction in visible fungal filaments in IVCM images early on after presentation, but then an increase in the proportion that had visible fungi in the IVCM images at the final visit.

Figure 3: Percentage of fungal keratitis patients with fungal filaments detected in HRT3 in vivo confocal microscopy imaging (Y-axis) at baseline and at each follow-up visit after enrolment (i.e. visit 1 at day 7 +/- 3 days; visit 2 at 14 days +/- 3 days or visit 3 at 21 days +/- 3 days, up to 37 days maximum) in the X-axis, for each clinical outcome.



Three of the patients whose ulcers had healed by the final visit (n=29) still had visible fungi in the IVCM images at that time point, as well as evidence of resolving stromal inflammation (i.e. stromal dendritiform cells as well as stellate cellular processes with no visible nuclei in 2, but no inflammatory cells in honeycomb or non-specific distribution visible in any), and normal keratocyte appearances in the stroma in all 3.

Cellular features associated with corneal perforation

In the univariable regression, no cellular morphologies in baseline or final visit IVCM images were significantly associated with perforation. In terms of change over time (i.e. absence of the cellular feature in baseline IVCM images but presence in the final visit images), two cellular features were associated with perforation in the multivariable analysis: dendritiform cells in the basal epithelial layer appearing in the final visit images (OR 3.31, 95% CI:1.00-10.97, p=0.05), or a normal keratocyte morphology becoming apparent in the final visit images (OR 4.49, 95% CI: 1.14-17.75, p=0.032).

Steroid use

Topical steroid use prior to presentation was not associated with any cellular features in IVCM images in the baseline or final visit, and did not have any statistically significant effect on change in any features between these two visits.

Discussion

Here we have described the cellular changes that occur in the cornea during the clinical course of human FK as observed with the HRT3 IVCM. We found that several cellular or structural morphologies in IVCM images were more likely in ulcers with a poor outcome. The stellate appearance of interconnected cellular processes with absence of visible nuclei, if present in the corneal stroma in baseline images, or development of this appearance in the final visit images, was an indicator of poor outcome. Although we did not conduct histological studies to confirm the cell type associated with this morphological appearance, we compared our images with those from previously published IVCM images and found that this appearance has also been reported post-LASIK[6], and after collagen-crosslinking[10]. The authors of these studies have postulated that these stellate interconnected cells are most

likely activated keratocytes, and the absence of visible nuclei may be due to loss of the nuclei secondary to a cellular event such as apoptosis,[6] or due to water retention causing change in refractive indices between intra-cellular components during corneal oedema, resulting in increased visibility of cellular processes and reduced visibility of the nuclei.[11] Immunohistochemical studies with TUNEL staining have confirmed that keratocyte apoptosis does occur in the human cornea in bacterial, fungal and *Acanthamoeba* keratitis.[12,13] The stellate inter-connected cellular appearance with absent nuclei that we observed in IVCM images, may be a useful warning sign of worsening keratitis - larger studies are required to confirm this finding and histological studies are required to elucidate the cell type corresponding to this morphology.

The presence of an inflammatory cell infiltrate forming a honeycomb distribution in IVCM images at the final visit was also associated with worsening ulcers in our study. A similar morphological appearance has been reported in a mouse model of corneal abrasion injury, where inflammatory cells have been observed to traverse along keratocyte processes in a honeycomb pattern using HRT3 IVCM live imaging (in the anaesthetized mouse), and the inflammatory cell type confirmed as the neutrophil in immunohistochemical examination of the same corneal tissue *ex vivo*.[14] Persistence of neutrophils has been associated with worse clinical outcomes and corneal perforation in BK and FK, most likely due to their ability to release tissue-damaging enzymes (e.g. MMP9) and pro-inflammatory cytokines (e.g. IL1B) that perpetuate the host inflammatory response.[15-17]

Another feature that was associated with worse outcome was the detection of fungal hyphae in final visit IVCM images. Persistence of fungi even at later stages of keratitis may be due to inadequate host mechanisms to clear the infection; one such mechanism may be through

production of molecules that are able to subvert the host immune response, e.g. alkaline phosphatase-1 secreted by *Aspergillus* sp. that is able to destroy host complement proteins C3 and C4.[18] Also, poor penetration of natamycin eye drops into the deeper cornea, and poor efficacy of this medication against some fungal species such as *Aspergillus* sp., may contribute to persistence of fungi in the deep cornea in late-stage keratitis.[19,20]

We found that the appearance of dendritiform cells in the basal epithelial layer or normal keratocyte morphology in final visit images, but absence in baseline images was associated with corneal perforation in our study. This implies that the fungi in these severe ulcers are able to grow through the cornea successfully with only a superficial immune response triggered in the basal dendritiform cells, and without triggering any response in the local keratocytes. Histological studies of corneal tissue excised at the time of corneal transplantation surgery in severe FK have shown that regions of the tissue with a high fungal load frequently do not have any inflammatory cell infiltration.[21] Possible reasons for this may be lack of penetration of natamycin into the deeper cornea, or the fungal subversion of the host response as outlined above. Corneal perforation may then occur in these cases due to fungal proteases damaging corneal collagens, including those in Descemet's membrane.[22,23]

The appearance of dendritiform cells in the stroma at the final visit, when not present at the baseline visit, was also associated with ulcer worsening. These cells may represent dendritic cells or macrophages that have been recruited to the ulcer towards the later stages of disease, or may also represent fibroblasts that are involved in wound repair at the site of the ulcer, as demonstrated in studies that have compared IVCM images with *ex vivo* histology of the same tissue.[24] Further comparative histological studies are required to identify the cell

type forming this morphological appearance in the IVCM images, in order to understand the role these cells may play in the worsening of disease.

One limitation of our study is that although we have identified IVCM cellular appearances, there are potentially multiple cell types that can form these morphologies. For example, cells with a dendritiform appearance in IVCM images may be macrophages or dendritic cells that reside within the cornea, but may also be bone-marrow derived myeloid cells that have migrated into the inflamed tissue.[25,26] Correlated images using both IVCM and immunohistochemistry within the same tissue sample are required in future studies in order to identify cell types and their morphology. Our study has focused on large ulcers and so findings are mainly applicable to moderate-to-severe keratitis; further research is required in ulcers at an earlier stages of presentation in order to identify whether the same cellular morphologies that we have found are also present in early disease.

Another limitation is that the HRT3 IVCM allows high resolution imaging of the living cornea, but with a limited field of view, and so requires multiple images to be taken of the ulcer margins to build up an overall picture of cellular changes in the whole ulcer.[1] As such an experienced operator is required so that the best set of images possible are captured. Also, an experienced observer is required to successfully identify the morphological features that we have described in our study, and so training would be required. We did not assess inter- or intra-grader agreement in this study, as only a single observer was used, however our prior report has shown there is variability in inter-grader agreement based upon grader experience. Hence further studies are required to not only validate IVCM image-based prognostic markers, but to also assess the diagnostic accuracy of graders in detecting these morphologies. A major limitation of IVCM at present is that the current techniques do not

allow exact re-imaging of the same cellular location in the cornea at future follow-up visits. In this study we have attempted to image as much of the ulcer as possible with multiple volume scans, to try to image as much of the ulcer as possible from epithelium to endothelium. Experimental methods are currently underway to combine anterior segment OCT techniques with HRT3 IVCM to attempt to be able to co-locate the imaging location within the cornea, and so future combined imaging modalities such as this may help to be able to serially assess pathological changes in the cornea over time.[27]

The alternative IVCM system available, Confoscan, provides a motorized lens that allows rapid imaging of the entire corneal ulcer quickly for less experienced operators, but will provide lower resolution images, with less detail of the keratocyte intracellular changes. However, use of the white light-based Confoscan system (Nidek technologies, Japan) in FK in high prevalence settings has in some studies allowed identification of resolution of inflammatory cell infiltrates and fungal filaments,[5] and so further research is required to assess morphological parameters in images from this system and whether they may correlate with clinical outcome. Future studies could also consider automation of the assessment of cellular features, such as cell counting to estimate keratocyte cell density, or size of dendritiform cells, or to assess the fungal filament load in each images. Automation using computational analysis would make such assessments much easier for the inexperienced observer, and also in the research setting since the number of total images from all volume scans are often too large to allow for manual cell counting or fungal filament tracing (>65,000 images screened in this study alone).

In summary, we have found that several cellular morphologies as detected with HRT3 IVCM may be associated with worsening versus healing ulcers. Since it can often be difficult to

monitor response to treatment with clinical examination alone, these specific IVCM features should be further investigated as possible prognostic biomarkers.

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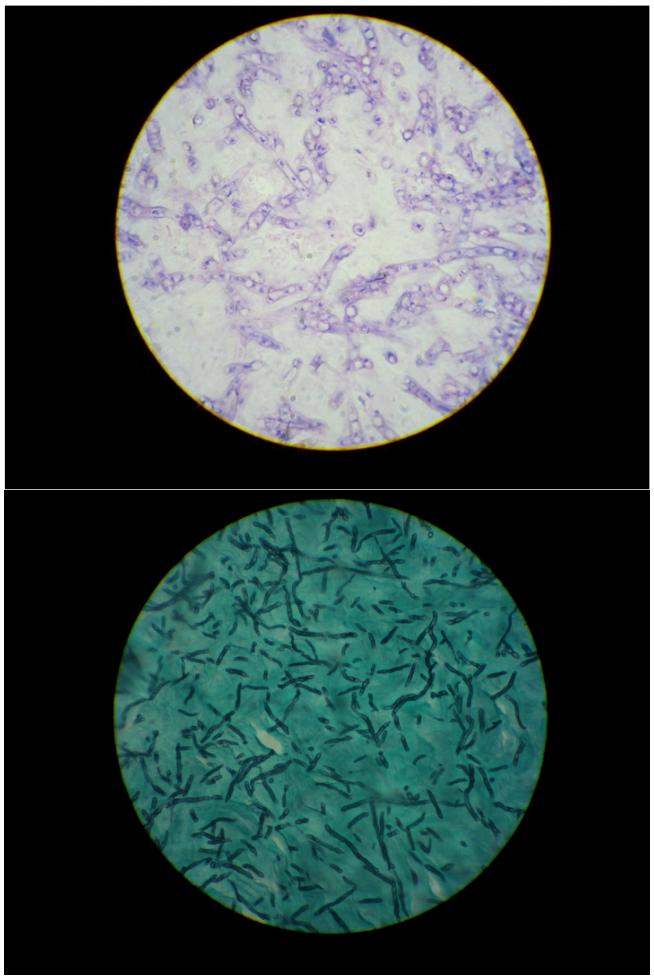
Chapter 8

Further Discussion, Overall Conclusions and Future Directions



En face sections of human fungal keratitis tissue stained with periodic acid Schiff (top image) and gomori methenamine silver (bottom image) to show details of fungal hyphae (histopathology performed by Dr. Shanthi and team, Dept. of Ocular Pathology, Aravind Eye Hospital, Madurai)

Overall Conclusions and Future Directions

Microbial keratitis remains a serious blinding condition, affecting millions of people worldwide. The poor visual outcomes from this disease are partly due to difficulty in ascertaining the causative organism early on, and ongoing tissue destruction due to host immune/inflammatory responses despite use of the correct antimicrobial agent. The aims of this PhD work were to improve knowledge of the biological processes involved in severe microbial keratitis as well as to elucidate whether the high-resolution imaging modality of IVCM could aid in diagnosis and management of this disease. We include here further discussion based upon each results chapter of this PhD thesis.

8.1 Epidemiology, Risk Factors & Clinical Outcomes in Severe MK in South India

In this epidemiological cohort study of moderate to severe MK in South India, we found that the majority of ulcers were culture-positive for fungi (i.e. 76%), with fewer bacterial, acanthamoebal or culture-negative ulcers overall. There may be several reasons for this finding. We chose to focus on large ulcers (stroma infiltrate ≥3mm) in this PhD study and it is possible that fungal ulcers were on average larger than bacterial ulcers. As Aravind Eye Hospital is a tertiary referral centre, many patients had received prior antibiotic treatment before presentation at the AEH Corneal Clinic (74% in our cohort) therefore bacterial ulcers may have been successfully treated and not presented as severe keratitis to be included in this study. Previous studies in South India have found that larger corneal ulcers are mainly caused by *Fusarium* sp. or *Aspergillus* sp.[1] diameter. Also, studies in Australia have associated severe MK (i.e. with vision loss) in tropical climates with environmental organisms, such as *Pseudomonas* sp. and fungi, rather than bacterial species such as *Staphylococci*.[2] In terms of the bacterial profile of organisms isolated, studies from Tamil Nadu have frequently found *Streptococcus pneumoniae* to be the most commonly isolated bacteria in corneal scrapes from MK of all sizes, closely followed by *Staphylococci*.[3, 4] However, it may be that more severe MK causing ulcers with infiltrate diameter ≥3mm may be more likely to be caused by *Streptococcus pneumoniae* rather than *Staphylococci* in this setting of AEH where this bacteria is already the more prevalent bacterial pathogen.

As such the findings of this study are applicable primarily to regions with a similar distribution of pathogenic organisms, climate and socio-economic status.

8.2 Diagnostic Accuracy of *In Vivo* Confocal Microscopy for Severe MK

Here we provide the first published report of a prospective study of the diagnostic accuracy of the laser-scanning HRT3/RCM IVCM for FK and AK.[5] Although we found a high sensitivity and specificity for the detection of both FK and AK[5], we also found that the accuracy was influenced by the experience of the person interpreting the IVCM images, as also shown by others.[6] There is a need to increase training for personnel involved in IVCM image grading, and one future direction following this PhD study would be to utilise the IVCM images to create a training guide for the detection of fungal filaments and *Acanthamoeba* cysts. Use of an image-set with pre-selected IVCM images that are representative of specific pathogens, interspersed with "negative controls" (i.e. culture-positive bacterial keratitis) would allow determination of the sensitivity and specificity of each individual grader in detecting fungi or *Acanthamoeba* cysts in future studies. The development of automated computational methods to detect fungal filaments[7] and *Acanthamoeba* cysts, with the use of artificial intelligence algorithms to improve accuracy is a focus for current and future research – discussed further in Section 8.5 below. Furthermore, use of IVCM imaging of the fellow eye could be utilised as a control however, this would provide misleading information in MK since past studies in patients with unilateral MK have found significant changes such as increased dendritiform cell counts, reduced endothelial counts and reduced sub-basal corneal nerve density in these patients' fellow eyes.[8-10]

In the study in Chapter 4 of this thesis, we have examined the sensitivity and specificity as well as inter-observer agreement in all patients with moderate-to-severe MK presenting to the Cornea Clinic in a real-life setting in South India. Prior prospective studies in this field also have a similar incidence of FK or AK in their study population.[11] Several studies, have utilized selected images from a selected population, thereby setting an artificially high rate of AK or FK, along with "control" images taken from culture-positive bacterial keratitis. In this way, these studies have selectively tested the diagnostic accuracy of observers from various countries, including the UK and US, in detecting fungi and *Acanthamoeba* cysts in these pre-selected patient populations. This provides a different type of methodology to assess diagnostic accuracy that heavily relies on the types of images selected and presented to the grader. Bias may be introduced into these studies if the selected images are not representative of the types of images that frequently present in the total population of MK patients. Prospective studies present all the images of the entire population of patients with MK attending the eye clinic, thus giving a better real-life estimation of diagnostic accuracy,

again using bacterial keratitis as the control. In the study in Chapter 4 of this thesis, we chose to study large ulcers (3mm or larger in diameter) since these frequently present with an atypical appearance and would therefore benefit from IVCM imaging to help in making the diagnosis. The diagnostic accuracy outcomes from our study may therefore be applicable predominantly to other regions where large ulcers present and are mainly caused by filamentous fungi. Prior studies have investigated the influence of disease prevalence in a population with the sensitivity and specificity of a diagnostic test.[12] These studies have found that higher disease prevalence is associated with higher sensitivity but lower specificity.[12] As such, further IVCM studies are required to prospectively assess the diagnostic accuracy in patients with all-size corneal ulcers in regions with low and high incidence of FK and AK.

Although we have shown that IVCM is indeed a valuable tool for the diagnosis of moderate-to-severe microbial keratitis, with results applicable to large ulcers in regions with similar socio-economic status and microbiologic profile of MK. At present the cost of the *in vivo* confocal microscope itself remains high, and so may not be affordable in the regions of the world where it is needed the most, i.e. developing countries in which the incidence of severe MK is highest and the distinction between BK and FK most frequently needs to be made.

8.3 IVCM Appearance of *Fusarium* & *Aspergillus* species in FK

Since *Aspergillus* sp. frequently have worse clinical outcomes, we set out to investigate whether any morphological differences, such as fungal filament branching angles, were detectable from IVCM images between this fungus and *Fusarium* sp. We did not find any significant difference in branching angle between the two fungal species, and did not identify any other differentiating features (such as dichotomous branching pattern or sporulation).[13] However, there is a theoretical possibility that fungal branch angle may differ depending on the severity of the ulcer. Future studies are required to investigate whether there is any significant difference between the branch angle in *Fusarium* and *Aspergillus* sp. in early stage corneal ulcers, measuring less than 3mm in diameter. Branch angle is affected by many different parameters, including topography of the tissue through which the fungal filaments are growing,[14] as well as the nutritional status of the fungus/nutrient availability of the surrounding tissue,[15] and both of these parameters may be affected by some of the tissue destruction that occurs in later stage corneal ulcers.

8.4 IVCM Cellular Features of BK, FK & AK

Analysis of the difference in cellular and structural changes in the cornea between organisms (i.e. in BK, FK or AK) did however show some specific changes. IVCM images of BK, predominantly caused by *Streptococcus pneumoniae* in this study, were more likely to have bullous changes in the corneal stroma. FK were more likely to have an inflammatory cell infiltrate (most likely to be neutrophils) in the anterior stroma, and *Aspergillus* ulcers in particular were associated with stromal dendritiform cells. AK were the most likely to have lost the normal appearance of keratocytes. All of these morphological findings give an important insight into the pathophysiology of disease. Future work to explore the findings of our study include correlative studies to assess the specific cell types that correspond to each morphological appearance in the IVCM images, i.e. using immunohistochemical techniques. Also, findings such as grouping of *Acanthamoeba* cysts in lines or clusters warrants further investigation using in vitro models to assess cyst coalescence in different environments, as well as further in vivo studies using IVCM imaging in patients with different stages of AK, in case this may indeed represent a prognostic marker.

8.5 Cellular Morphological Changes associated with Clinical Outcome in FK as detected with Laser Scanning IVCM

Daily slit lamp examination of bacterial, fungal and *Acanthamoeba* keratitis can often show very little macroscopic change in the morphology of the ulcer, and so it can sometimes be difficult for the clinician to determine whether there is a therapeutic response based on clinical features alone. In the final IVCM study of this PhD we assessed serial IVCM images obtained over the course of FK to look for parameters that may have been associated with either good or poor outcome. We found that images taken in the first visit that showed loss of visible nuclei in the interconnected cellular network (probably the keratocyte network) anywhere in the cornea were associated with poor outcomes. This for the first time provides a morphological feature that may have prognostic value in patients presenting with FK to the clinic. Further studies are currently underway (with Prof. Luthert at the Dept. of Pathology, the Institute of Ophthalmology) to be able to confirm the specific cell types corresponding to these IVCM morphologies through the use of immunohistochemical stains (IHC) in corneal tissue excised at corneal transplant surgery in study participants for whom correlative IVCM imaging is available. It may be possible to apply more sophisticated multi-colour IHC methods to stain multiple molecules within the same tissue, thus allowing

localisation of multiple molecules as well as incorporating stains to identify the cellular source.[16]

We also detected that fungal filaments disappeared at earlier time points in fungal ulcers that were healing/improving based on slit-lamp appearances, but persisted until later visits in worsening ulcers. Previous studies have used days to ulcer re-epithelialisation as a measure of healing,[17] however, we were unable to use this in our study of fungal keratitis as we examined ulcers weekly and so would only be able to estimate healing to the nearest 7 days, and also many ulcers had healed epithelium but persistent enlargement of the stromal infiltrate (i.e. stromal abscess). Hence ulcer stromal infiltrate size was used as a measure of ulcer resolution in our study.

Although, it is difficult to re-image in exactly the same corneal anatomical location using HRT3 IVCM at each follow-up visit, assessing the fungal filament density/quantity at each visit in regions of the corneal stroma may be a method by which the therapeutic response to antifungal treatment could be quantitatively estimated. Three-dimensional reconstruction of sequential HRT3 IVCM images within a volume scan is possible.[18] Automation of this process is limited due to patient eye movement between adjacent images, but manual registration of these images is possible. Computerised methods have been developed for quantitation of corneal nerves, so it may be theoretically possible to apply some of these methods to fungal filament quantitation in future studies.[19] Use of the HRT3 IVCM imaging system in the anaesthetised mouse (thus minimising eye movement between sequential images), followed by exploration of the same corneal tissue using *ex vivo* IHC has already provided valuable information about the molecular mechanisms underlying the movement of neutrophils within the corneal collagen structure after abrasion injury.[20] Further research could therefore use this dual approach of *in vivo* imaging with *ex vivo* IHC (or other molecular techniques) to explore specific molecular mechanisms associated with certain cell types within the cornea at different stages of infection in MK. Using computerised techniques to join together imaging data and molecular data by their tissue location, a "map" can be created of an entire tissue or organ showing the host-pathogen interaction and how it varies by spatial location. Garg *et al* recently used this cartographic method to combine computed tomography (CT) images of the lungs of a cystic fibrosis patient with *Pseudomonas* infection along with microbiome (16S rRNA sequencing) and metabolomics data (mass spectrometry identifying host and pathogen molecules as well as specific antibiotics).[21] They showed the regional variation of *Pseudomonas* sp. (and other bacteria), metabolic molecules (including those

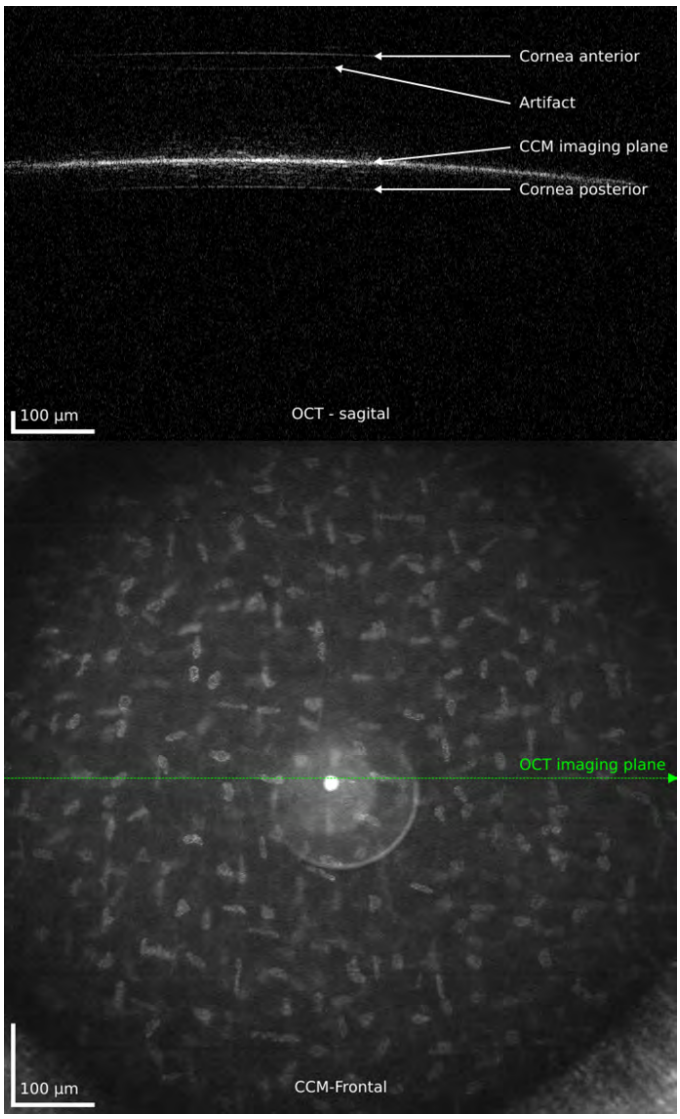
produced by *Pseudomonas* sp.) and how these varied with antibiotic penetration into the tissue.[21] Applying such a technique to a model of corneal infection by combining IVCM data and molecular data would allow us to explore the host-pathogen response to be able to answer specific "cartographic" questions such as why "hotspots" might develop in certain regions of the cornea resulting in localised perforation.

A limitation of this study is that with the HRT3/IVCM system, we are at present not able to know the specific location from which an image is obtained within the X-Y-Z-axis in the cornea, hence it is impossible to be able to re-image the exact same location of the cornea at follow-up visits. In IVCM images obtained at the moment, we are only able to identify whether the images were taken in the plane of the epithelium, stroma or endothelium by observing the morphology of the cells within the image and looking at the corneal pachymetry measurement (which relies upon the operator to accurately reset this when imaging commences at the superficial-most images obtained at the corneal surface), hence limiting repeatability of imaging. Since Optical Coherence Tomography (OCT) imaging provides a method by which a wider-field (but lower-resolution) image of the cornea can be obtained showing all layers of the cornea (i.e. axial imaging rather than the en face imaging of the cornea with IVCM), a current advance in IVCM research is the development of a hybrid OCT-IVCM machine, that can allow HRT3 IVCM imaging with the RCM lens to maintain the high-resolution IVCM image (805μm x 805μm image size in X-Y axis), but to a wide-field view of the entire corneal diameter in a OCT line-scan image in the Z-axis (805μm x 1919μm image size), this allows the operator to know the specific location within the cornea at which the IVCM image is obtained. This information would therefore allow the operator to be able to re-image the same location in the cornea serially over time, using both the IVCM and spectral domain OCT simultaneously (see Figure 1 for example of the image obtained).[22]

Another limitation in the reproducibility of all of the IVCM studies in this thesis, is the need for a highly trained operator to obtain high quality images of the corneal ulcer, and a highly trained grader to interpret the images. Possible solutions to these issues are to create protocols for image acquisition to allow technicians to be able to obtain high quality images of corneal ulcers. Others have developed grid systems positioned behind the IVCM to allow the patient to systematically fixate using the fellow eye, thus enabling a trained technician to obtain images of the entire cornea, e.g. for mapping of the corneal sub-basal nerve plexus.[23] Alternatively, use of a non-contact lens version of the Rostock corneal module has been

used in some research studies, and allows a less experienced operator to be able to obtain HRT IVCM images of the cornea.[24] However, this provides a larger field of view (500μm x 500μm) with less magnification (50x) compared to the contact RCM (63x) so would be less helpful for the identification of small pathogens such as *Acanthamoeba* cysts where higher magnification would provide a better image.

Figure 1: Corneal stromal images obtained with the experimental hybrid HRT3/RCM In Vivo Confocal Microscope (IVCM) and Spectralis OCT2. The Spectralis OCT2 image (upper image) shows the corneal confocal microscopy imaging plane, and the IVCM image obtained simultaneously is shown in the lower image.[22]



Automated methods for image analysis have been used in other aspects of ophthalmology for image analysis (e.g. in retinal screening photos for diabetic retinopathy) and in theory automated methods with the addition of machine learning methods could also be used for recognition of key features such as presence of fungal filaments in IVCM images.[7] However,

much research is required in this field still, since other linear branching structures may be present in the cornea that resemble fungal filaments (e.g. corneal nerves), or round structures that resemble *Acanthamoeba* cysts (e.g. white blood cells) and so caution is required in the interpretation of results from an automated system that may produce false positives if these structures are wrongly classified as pathogenic organisms.

8.6 References

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Appendix 1a: LSHTM Ethics Committee Approval

13 February 2012

Jaya Chidambaram

Dear Jaya

Study Title:	**Studies of the diagnosis & pathophysiology of corneal necrosis in severe microbial keratitis**
LSHTM ethics ref:	**6118**
Department:	**Infectious and Tropical Diseases**

Thank you for your email of 13 February responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*	
LSHTM ethics application	n/a		
Protocol	V1.0	13/02/12	
Information Sheet	V1.0	13/02/12	
Consent form	V1.0	13/02/12	

After ethical review

Any subsequent changes to the application must be submitted to the Committee via an E2 amendment form.

Yours sincerely,

Professor Andrew J Hall
Chair

Appendix 1b: Aravind Institutional Review Board Ethics Committee Approval Letter

ARAVIND MEDICAL RESEARCH FOUNDATION

8th October 2011

To

Dr.N.Venkatesh Prajna
Chief-Cornea Services
Aravind Eye Hospital
No.1, Anna Nagar
Madurai

PRESIDENT
Dr. P. Namperumalsamy MS, FAMS
VICE PRESIDENT
Dr. G. Natchiar MS, DO
SECRETARY CUM TREASURER
Dr. R. Kim DO, DNB
DIRECTOR - RESEARCH & IMMUNOLOGIST
Dr. VR. Muthukkaruppan MSC, Ph.D

Dear Dr. Venkatesh Prajna,

The Institutional Review Board of Aravind Eye Care System reviewed and discussed your application to conduct the clinical trial entitled **"Studies in the pathophysiology and management of corneal necrosis in severe microbial keratitis"** on 8th October 2011 at 3.00Pm at D.GVERI Conference hall.

The following members of the ethics committee were present at the meeting.

Ms.Shobhana Ramachandhran, M.A [redacted]	Managing Director TVS Sri Chakra Ltd Madurai	Chairman CHAIRMAN Institutional Review Board/Ethical Committee Aravind Eye Hospital Madurai-20
Dr.Lalitha Prajna, MD DNB	Chief Microbiologist Aravind Eye Hospital Madurai	Member-Secretary
Dr.C.Srinivasan, M.Sc.,Ph.D [redacted]	UGC Emeritus Professor Department of Chemistry Madurai Kamaraj University New No. 2/249, 7th Street Kalvinagar, Rajambadi Madurai	Member MEMBER Institutional Review Board/Ethical Comm Aravind Eye Hospital Madurai-20
Dr.T.S.Chandrasekaran, MS DO	Ophthalmologist Gandhi Nagar, Madurai	Member
Dr.L.Thayumanavan, MD, DM	Sr.Consultant Gastroenterologist Vadamalayan Hospital, Madurai	Member

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1, Anna Nagar, Madurai 625 020, Tamil Nadu, India; Phone: 0452-435 6550; Fax: 91-452-253 0984
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ARAVIND EYE CARE SYSTEM

ARAVIND MEDICAL RESEARCH FOUNDATION

Mr.M.Mariappan, B.Sc., BL [redacted]	Advocate 355, Anna Nagar Madurai	Member MEMBER Institutional Review Board/Ethical Committee Aravind Eye Hospital Madurai-20
Mr.M.Senthilkumar, M.A, BL [redacted]	Advocate 32, Sindanayalar Nagar Karuppayoorani East Madurai	Member [redacted] MEMBER Institutional Review Board/Ethical Committee Aravind Eye Hospital Madurai-20
Mr.R.Raja Govindasamy, M.A., M.A	Former Principal Thiagarajar College 169-1, 2nd cross street, I Main Road, Gomathipuram Madurai	Member
Dr.R.Muralidhar, MD (Ophthalmology)	Medical Officer Paediatric Opthalmology Aravind Eye Hospital, Madurai	Member

We approve the trial to be conducted in its presented form.

None of the Investigators and the members of the study team involved in the study participated in deliberations and voting of the said study during the meeting.

The Institutional Review Board expects to be informed about the progress of the study, any SAE occurring in the course of the study, any changes in the protocol and patient information/informed consent and asks to be provided a copy of the final report.

This is to certify that the Ethical Committee operates as per ICH GCP, Schedule Y and ICMR guidelines.

Yours Sincerely,

[redacted]

Shobhana Ramachandhran
Chairman – Institutional Review Board

1, Anna Nagar, Madurai 625 020, Tamil Nadu, India; Phone: 0452-435 6550; Fax: 91-452-253 0984
E-mail: amrf@aravind.org; www.aravind.org

Appendix 2a: Study Participant Information Leaflet (English)

Studies in the management of corneal necrosis in severe microbial keratitis

AEH / LSHTM Microbial Keratitis Research Programme
Aravind Eye Hospital & London School of Hygiene & Tropical Medicine

1. What is this study about?

Infection of the clear part of the front of the eye (the cornea) by bacteria or fungi is called microbial keratitis and is a major cause of blindness in India. A scratch in the cornea allows infection to enter the surface of the eye, and a corneal ulcer begins. As the infection progresses, the cornea can become severely affected, and sometimes can even become thinned. Sometimes this thinning continues to happen even after starting the correct eyedrops to treat the infection. Currently, this disease process is only poorly understood and the existing treatments for it are only partially effective. We believe that the reason some people develop thinning of the cornea is because of the nature of the way their bodies defence systems respond to this infection. The study will help to find out more about the way the body fights the infection in the cornea.

2. Whose help do we need?

We need the help of some 500 adults attending Aravind Eye Hospital Cornea Clinic.

3. What will we ask you to do?

If you agree to participate we will ask you several questions about yourself, such as age and about your general health. You will be recruited into a three week study in which we would examine your eyes regularly. If your infection is severe, we may advise that you stay in hospital for daily review for the first few days. You will be treated with standard antibiotic or antifungal eyedrops regularly for three weeks. Aravind Eye Hospital has its own pharmacy which has available at all times all of the antibiotic and antifungal medications required to treat your eye infection. We will examine your eyes and collect one swab sample from the infected area of the cornea by gently rubbing it on the surface of the eye. We will also collect a sample of tears from the surface of the eye. At the beginning of the study and again at the end of weeks 1, 2 and 3, we would use a special microscope to look at the very fine detail of the surface of the eye. We will also take a photograph of the eyes on each occasion.

4. What benefits are there to taking part in the study?

1. It is only poorly understood how this disease causes blindness. You will be helping us to answer this question.
2. We will check the general health of your eyes and refer you to receive appropriate eye care through the Eye Clinic in Madurai or an alternative clinic if it is more convenient.

5. Are there any risks caused by taking part in the study?

There is no known risk from taking swab or tear film samples, or from taking images with the special microscope. All of these tests have been done before in many studies conducted elsewhere in the world.

6. What tests will we do on the sample?

The eye swab samples will be tested in laboratories in Aravind Eye Hospital, Madurai, and London in various ways to try to help improve the understanding of microbial keratitis. They will be tested to find out what type of infection (bacterial or fungal) has caused the problem. We will also study how you are responding to try to understand better how it our immune systems fight the infection and how this might result in scarring problems. We will test your sample to look for some specific genes and proteins to find out which ones might be involved in bringing about more severe corneal ulcers. Eye swabs will be stored until all the samples have been collected, which may be up to two years, and then all samples will be tested. After testing, the residual eye swab samples will be archived for up to 10 years, in case any additional analysis of the gene expression or protein is needed.

7. What will happen to the records and photos we keep about your eye?

All the information we collect will be kept confidential. It will be kept securely and only the people organising the study will have access to it.

8. Do I have to take part in this study?

You do not have to take part in this study, it is entirely voluntary. If you do not want to participate in the study, this will not affect treatment of your eye infection in any way and you will continue to receive standard treatments for your infection as a patient of the Cornea Clinic at Aravind Eye Hospital.

9. Who is doing this study?

This project is a collaboration between the Aravind Eye Hospital, India and the London School of Hygiene & Tropical Medicine, UK. The study will be coordinated in India by Dr Jaya Chidambaram. The eye examinations will be conducted by a small team of eye doctors and eye nurses.

If you have any questions please ask us:
Dr. Jaya Chidambaram: Email: jaya.chidambaram@lshtm.ac.uk
Dr. NV Prajna: Email: Prajna@aravind.org

Appendix 2b: Study Patient Information Leaflet (Tamil)

தகவல் படிவம்

படிவம் ஆ. நுண்ணியல் கிருமிகளால் கருவிழிச் சிதைவைப் போக்கும் ஆய்வு : இந்த ஆய்வு எதைப் பற்றியது:

இந்தியாவில் கண் பார்வை இழப்பிற்கு, கருவிழியில் பாக்டீரியா மற்றும் புஞ்சை (காளான்) எனும் நுண்கிருமிகளால் ஏற்படும் தொற்றுநோய் ஒரு முக்கிய காரணமாக உள்ளது. கருவிழியில் ஏற்படும் ஒரு சிறு சிராய்ப்பு அல்லது காயம் மூலமாக நுண்கிருமிகள் கருவிழிக்குள் புகுந்து பின் கருவிழிப் புண் ஏற்படுகிறது. நுண்கிருமிகள் மேலும் தொடர்ந்து விருத்தியடைவதால் கருவிழி சிதைவடைய நேரிடுகிறது. பிறகு கருவிழி மிக மெல்லிய தன்மை அடைகிறது. சில சமயங்களில் இந்த கிருமிகளை அழிப்பதற்கு ஏற்ற சரியான கண் மருந்தை செலுத்தியும் கருவிழியின் இந்த மெலிவைத் தடை செய்ய முடிவதில்லை. இன்று வரையிலும் இந்த கருவிழி நோயை சரியாக புரிந்து கொள்ள முடியாததால், தற்சமயம் கிடைக்கும் சிகிச்சை முறைகள் ஓரளவுதான் குணப்படுத்துகின்றனவே தவிர, முழுமையாகக் குணப்படுத்த முடியவில்லை. கருவிழியின் மெலிவு/தேய்மானத்துக்குக் காரணம், நோயாளிகளின் உடலில் உள்ள நோய் எதிர்ப்புத்தன்மையையும் பொறுத்து உள்ளது என நாங்கள் கருதுகிறோம். ஆகவே, நோயாளிகளின் நோய் எதிர்ப்புத்தன்மை எவ்வாறு இந்த நோயை கட்டுப்படுத்துகிறது என்பதை கண்டறியவே இந்த ஆய்வு.

எங்களுக்கு யார் உதவி தேவை:

அரவிந்த் கண் மருத்துவமனையின் கருவிழிப் பிரிவிற்கு வரும் 500 கருவிழி தொற்றுநோயினால் பாதிக்கப்பட்டவர்கள்.

தங்களின் பங்களிப்பு :

இந்த ஆய்வுக்கு சம்மதித்தால் உங்கள் உடல்நிலை பற்றிய சில கேள்விகள் கேட்கப்படும். பிறகு நீங்கள் 3 வார ஆய்வு மற்றும் சிகிச்சைக்கு சேர்த்துக்கொள்ளப்படுவீர்கள். தகுந்த மருத்துவ உதவி உங்களுக்கு பரிந்துரைக்கப்படும். மேலும் தேவைப்பட்டால் உங்களை உள்நோயாளியாக அனுமதித்து இந்த சிகிச்சை தொடரப்படும். உங்கள் கண்களைப் பரிசோதித்து கருவிழியின் முன்பகுதியிலிருந்து கிருமிகளை துடைப்பான் மூலம் சேகரிக்கப்படும். உங்கள் கண்களின் புகைப்படமும் எடுக்கப்படும்.

இந்த ஆய்வினால் கிடைக்கும் பலன் :

1. இந்த கேள்விக்கு விடை காண உங்கள் ஒத்துழைப்பு உதவும்.
2. உங்கள் கண்கள் முழுமையாக சோதிக்கப்படும்.

இந்த ஆய்வில் கலந்து கொள்வதால் ஏதேனும் சிக்கல் உண்டா?

உங்கள் கண்களிலிருந்து துடைப்பான் மூலம் கிருமிகளை எடுப்பதாலும், மைக்ரோஸ்கோப்பில் புகைப்படம் எடுத்தாலும் எந்தச் சிக்கலும் இல்லை. இவை எல்லாம் உலகத்தின் பல்வேறு இடங்களில் முன்பே செய்யப்பட்டுள்ளன.

இந்த ஆய்வில் என்னென்ன சோதனைகள் செய்யப்படும்

உங்கள் கண்களிலிருந்து எடுக்கப்பட்ட துடைப்பான்களை ஆய்வுகூடத்தில் பல்வேறு சோதனைகளுக்கு உள்ளாக்கி ஆய்வதன் மூலம், கருவிழி நோயைப் பற்றி மிக நன்றாகப் புரிந்து கொள்ள இயலும். அந்த துடைப்பான்களைச் சோதிப்பதன் மூலம், கருவிழி நோய் வரக் காரணம் நுண்ணியல் கிருமிகளா, அல்லது புஞ்சை, காளான்களா என்பதைக் கண்டறிய இயலும். அத்துடன் இந்த நோய் வந்தபின் உங்கள் தடுப்புத்திறன் எப்போது வேலை செய்கிறது என்பதையும் ஆய்வு செய்ய முடியும். கருவிழிபுண் ஆறும் போது வடு ஏதேனும் உண்டாகிறதா என்பதையும் கண்டறிய முடியும். இந்தத் துடைப்பான்களை ஆய்வு செய்வதன்

மூலம், சில புரதச் சத்துக்களையும் சில மரபணுக்களையும் கண்டு, அவற்றில் எதன் மூலம் கருவிழிப்புண் முற்றிய நிலையை அடைகிறது என்பதை அறியலாம். கண்களிலிருந்து எடுக்கப்படும் துடைப்பான்களை போதிய எண்ணிக்கை வரும் வரை சேமித்து வைக்கப்படும். இதற்கு சுமார் இரண்டாண்டுகள் வரை ஆகலாம். அதன்பின் அவை மொத்தமாக சோதிக்கப்படும். ஏனெனில் பிற்காலத்தில் புதிய மரபணு புரதச்சத்துகளை ஆய்வு செய்ய வேண்டி வரும் பட்சத்தில் இவை அதற்காக உபயோகப்படுத்தப்படும்.

உங்களது பதிவேடுகளும், புகைப்படங்களும், நாங்கள் சேமித்த வைக்கும்போது என்ன ஆகும்:

அவை அனைத்தும் பத்திரமாகப் பாதுகாத்து வைக்கப்படும். இந்த ஆய்வில் சம்பந்தபட்டவர்களைத் தவிர மற்றவர்களுக்கு இவற்றைப் பார்க்க அனுமதி கிடையாது.

நீங்கள் இந்த ஆய்வில் பங்கேற்கத்தான் வேண்டுமா?

இந்த ஆய்வில் நீங்கள் பங்கேற்பது உங்கள் சொந்த முடிவு. இந்த ஆய்வில் நீங்கள் சேர வேண்டும் என்பது கட்டாயமில்லை. இந்த ஆய்வில் சேராததினால் உங்கள் சிகிச்சை முறையில் எந்த வேறுபாடும் இருக்காது. அரவிந்த் கண் மருத்துவமனையின் தரமான சிகிச்சை முறை உங்களுக்குத் தொடர்ந்து கிடைக்கும்.

இந்த ஆய்வை நடத்துபவர்கள் யார்

இந்தியாவில் அரவிந்த கண் மருத்துவமனையும் இலண்டனில் உள்ள சுகாதாரம் மற்றும் வெப்பநிலை மருத்துவபல்கலைக் கழகமும் சேர்ந்து இந்த ஆய்வை நடத்துகிறார்கள். இந்தியாவில் இந்த ஆய்வை இணைந்து நடத்துபவர் டாக்டர் / மருத்துவர் ஜெயா சிதம்பரம்,

கண் மருத்துவர்களும் மற்றும் செவிலியர்களும் அடங்கிய ஒரு சிறுகுழு உங்களது கண்களை சோதனை செய்வார்கள்.

வேறு ஏதேனும் கேள்விகள் இருந்தால், கீழ்க்கண்ட மருத்துவர்களை அணுகவும்

1. மருத்துவர் : ஜெயா சிதம்பரம் e mail : Jaya.Chidambaram@Lshtm.ac.uk
2. மருத்துவர் : N. வெங்கடேஷ் பிரஜ்னா e mail : Prajna@aravind.org

<u>தொடர்புக்கு / Contact</u>

தொலைபேசி எண் : 0452 4356100 Extension-104, or cellphone no. 7598029150

க.சோபியா & டாக்டர்.ஜெயா சிதம்பரம் / Ms. G. Sophia & Dr. Jaya Chidambaram

அரவிந்த் கண் மருத்துவமணை / Aravind Eye Hospital,மதுரை / Madurai – 20.

Appendix 3a: Study Consent Form (English)

Studies in the diagnosis and pathophysiology of corneal necrosis in severe microbial keratitis

AEH / LSHTM Microbial Keratitis Research Programme
London School of Hygiene & Tropical Medicine

Name: …… MR No. : ……

Study Reference Number: | | | | |

I have read / had explained to me the information about the research study. I understand what will be involved in taking part in the study.

…….. has answered all my questions about the study.

PART A
I agree to take part in this study and that the sample from my eyes can be used for the study that has been explained to me.

Signed / Thumbprint…….. Date……..

Please tick one of the following boxes and sign below again

PART B
☐ I agree that any sample (DNA/RNA/protein), taken from my eyes, which has not been used in this initial study, can be stored and used for related studies in the future.

☐ I do not wish that any remaining sample (DNA/RNA/protein) taken from my eyes can be stored for use in future related studies and it should be destroyed.

Signed / Thumbprint…….. Date……..

I have explained the purpose of the study to the above subject and I am satisfied that he/she willingly agrees to participate

Signed…….. Date……..

Name:……..

In the event that the patient cannot read the above information, an additional witness is required:
I have witnessed the explanation and informed consent to this study of above named patient

Signed…….. Date……..

Name:……..

Appendix 3b: Study Consent Form (Tamil)

ஒப்புதல் படிவம்: கருவிழி புண்களை அல்சர், நுண்ணியல் கிருமிகள் எவ்வாறு பாதிக்கின்றன என்பதை பற்றி ஆய்வு

MR.No :

பெயர்/Name : _____

ஆய்வின் பதிவி எண் : | | | | |

நான் ஆய்வு படிவத்தில் உள்ள விவரங்களை படித்து தெரிந்து கொண்டேன்/ பிறரால் தெரிவிக்கப்பட்டேன். இந்த ஆய்வில் என் பங்கு மற்றும் உதவி எந்தளவு அடங்கியுள்ளது என்பதை பற்றி அறிந்துகொண்டேன்.

_____ அவர்களால் இந்த ஆய்வை பற்றி நான் கேட்ட அனைத்து கேள்விகளுக்கும் விளக்கம் கூறபட்டது

பகுதி அ:

என் கண்ணிலிருந்து ஆய்விற்குத் தேவையான சாம்பிள்களை எடுத்துக் கொள்ளவும் அதனை இந்த ஆய்விற்கு பயன்படுத்தவும் சம்மதிக்கிறேன். என்னுடைய சாம்பிள்கள் இந்த ஆய்விற்கு மட்டுமே பயன்படுத்தப்படும் என்று கூறியுள்ளார்கள்.

கையொப்பம்/கைரேகை _____ தேதி _____

கீழே உள்ள சிறுகட்டத்தினுள் அடையாளக் குறியிடவும்.பின் கையொப்பமும் இடவும்.

பகுதி ஆ:

□ என் கண்ணில் இருந்து DNA/RNA/Protein எடுக்கப்பட்ட சாம்பிள்களை முதல்நிலை ஆய்விற்கு பயன்படுத்தாமல் சாம்பிள்களை சேமித்து வைத்திருந்து பின்னர் இதற்கு இணையான ஆய்விற்கு பயன்படுத்த சம்மதிக்கிறேன்.

□ இந்த ஆய்விற்காக என்னிடம் இருந்து எடுக்கப்பட்டுள்ள சாம்பிள்களை DNA/RNA/Protein இந்த ஆய்விற்கு பயன்படுத்தி மீதம் உள்ள சாம்பிள்களை பின்னர் நடத்த இருக்கும் ஆய்விற்கு பயன்படுத்த நான் ஒப்புதல் தரவில்லை.

கையொப்பம்/கைரேகை _____தேதி _____

இந்த ஆய்வில் பங்கு கொள்ளுபவருக்கு ஆய்வு படிவத்தை வாசிக்க முடியாதவராக இருந்தால் கூடுதலாக ஒரு சாட்சி நபர் தேவைப்படும்.

நான், ஆய்வில் பங்கேற்கும் நபரிடம்/நோயாளியிடம் இந்த ஆய்வின் தகவல் படிவம் பற்றியும் ஒப்புதல் படிவம் பற்றியும் விளக்கம் கொடுத்ததை கண்டு அறியப்பட்டேன்.

கையொப்பம்/கைரேகை _____தேதி _____

பெயர் _____

நான் இந்த ஆய்வின் நோக்கத்தையும் மற்ற விவரங்களையும் தெளிவாக ஆய்வில் பங்கேற்றவருக்கு விளக்கம் கொடுத்தேன். அவன்/அவள் இந்த ஆய்வில் பங்கு கொள்ள விரும்பி சம்மதித்தனர்.

கையொப்பம்/கைரேகை _____தேதி _____

பெயர் _____

Appendix 4: Cohort Study Data Collection Form

Baseline Study Form

Eligibility Criteria:

Please tick boxes that apply. **If any of the exclusion criteria box is tick marked then the subject is not eligible.**	
Inclusion Criteria: ☐ Adults aged 18 years or over ☐ Presence of acute suppurative corneal ulcer with ALL of the following clinical signs: ☐ Corneal epithelial ulceration ☐ Corneal stromal infiltrate ≥3mm ☐ ≥1/3 depth of the corneal stroma is ulcerated or infiltrated ☐ Evidence of acute inflammation (injection / cells in AC / hypopyon) ☐ Consent to take part in the study ☐ Willingness to attend for follow up visits up to 3rd week after enrolment	**Exclusion Criteria** ☐ Age below 18 years ☐ Perforation of corneal ulcer at presentation: defined as a full thickness corneal ulcer, which extends into the anterior chamber. These findings may also be present: ☐ Flat anterior chamber ☐ Focal irido-corneal adhesion, eg iris plugging corneal ulcer at perforation site ☐ Positive seidel test ☐ Probable herpetic corneal disease on history or examination findings, eg ☐ History of Herpetic stromal keratitis or dendritic ulcers ☐ Dendritic ulcer ☐ Geographical ulcer ☐ VA worse than 6/60 in fellow eye ☐ Not willing to participate

ICF signed ☐Yes ☐No

Demographic Details:

Patient Name		MR No.			
Today's Date	__/__/____ d d m m y y y y	Study No.			
Phone. No.		Visit No.	1(Baseline)		
Address					
Date of Birth	__/__/____ d d m m y y y y	Age (years)		Sex 1 = Male 2 = Female	

	Options	**Answers**
Occupation	1= Manual Labour -Agriculture (Farmer, Agriculturalist, shepherd, landscaper, etc.) 2= Manual Labour – Non-agriculture (Construction, Mason, Wood cutter, Electrician, Millworker, etc.) 3= Driver 4= Semi-skilled/Skilled Labour 5= Business/Trade 6= Student 7= Unemployed/Not working 8= Service/Clerical Work 9 =Domestic Work (house maker, etc.) 10=Retired 11=Professional Work 12 =Other	
Education	0=No education 1=1st Std to 5th Std 2= 6th Std to 12th Std 3= Undergraduate degree 4=Postgraduate degree	
Have you ever smoked	1= Yes ; 2= No	
• If yes, how many years		
• Do you currently smoke	1= Yes ; 2= No	
Have you used tobacco	1= Yes ; 2= No	
• If yes, how many years		
• Do you currently smoke	1= Yes ; 2= No	

History:	Options	Answers
Affected Eye	1 = Right ; 2 = Left ; 3 = Both	
No. of days since onset of symptoms (pain/redness)?		
History of Trauma?	0 = No ; 1 = Yes	
If yes, Object of injury	1 = Vegetative Matter 2 = Wood/Tree branch/Firewood stick 3 = Assault 4 = Animal-related injury 5 = Metal 6 = Finger nail 7= Other (specify)	
Did you see another	1 = Yes ; 2 = No	

healthcare provider before Aravind? If **yes**, Whom?	1= Aravind Centre 2= General Physician 3= Refractionist 4= Native Medicine Healer 5= Non-Aravind ophthalmologist 6= Pharmacist 7= Other – specify:	
How many days after onset did you see the healthcare provider?		
Any reason for delay in coming to ophthalmologist?		
Native / Ayurvedic / Homeopathic Medicine used for this ulcer? Y/N • If yes, Plant based? Name of medicine For how many days? • Others specify For how many days?	1 = Yes ; 2 = No	
	1 = Yes 2 = No	
Contact lens use? If **yes**, No. of hours/day usage & name of lens/cleaning solution	1 = Yes 2 = No	

Any Past ocular history?	**Right Eye**	**Left Eye**
	Y/N?	**Y/N?**
• What diagnosis? • What treatment or Surgery? • Date • Previous corneal transplant?		

Corneal Ulcer Treatment Before Aravind

Name of the drug (99=not applicable)	Times/day	Dosage	Route	Start Date	Stop Date	Treatment Code

Any other eye drop medications being used, eg for Glaucoma?

Name of the drug (99=not applicable)	Times/day	Dosage	Route	Start Date	Stop Date	Treatment Code

General Medical History	Options	Answers	Other Specifications:
Any general health problems / hospitalisations / surgery?	1 = Yes 2 = No		Please specify below:
TB?	1 = Yes 2 = No		If yes – pls specify date of onset, treatments
Leprosy?	1 = Yes 2 = No		If yes – pls specify date of onset, treatments
Diabetes?	1 = Yes 2 = No		If yes – pls specify: on insulin or non-insulin dependent?
	Blood Glucose Level HbA1C value		
Any history of cancer?	1 = Yes 2 = No		If yes – pls specify
Rheumatoid arthritis?	1 = Yes 2 = No		If yes – pls specify date of onset, treatments
Ever tested HIV +ve or AIDS diagnosed?	1 = Yes 2 = No		If yes – pls specify
Any drug / eyedrop allergy?	1 = Yes 2 = No		Please specify:

Medication History

Name of the drug (99=not applicable)	Total daily dose	Route (tablet, ointment etc.)	Reason for Taking Drug	Start date	Stop date

DAP Study: Slit Lamp Examination

M.R. No.		**Patient Name**	
Study No.		**Today's Date**	_ _ /_ _ /_ _ _ _ d d m m y y y y
Visit No.	1(Baseline)	**Doctor Name**	

LogMAR Visual Acuity	**Right eye**	**Left eye**
Uncorrected		
Pinhole / BCVA		

	Options	**Answers**	
Affected Eye	1 = Right eye 2 = Left eye		
Eyelids		**Right eye**	**Left eye**
Normal?	1 = Yes ; 2 = No		
Lid swelling	1 = Yes ; 2 = No		
Trichiasis involving cornea?	1 = Yes ; 2 = No		
Lagophthalmos?	1 = Yes ; 2 = No		
No. of mm of lagophthalmos			
Good Bell's phenomenon?	1 = Yes ; 2 = No		
Entropion	1 = Yes ; 2 = No		
Ectropion	1 = Yes ; 2 = No		
Tear duct blocked?	1 = Yes ; 2 = No		
Cause for VA <6/80 in unaffected eye?	1 = Right eye 2 = Left eye		
Other (99), pls specify:			

Corneal Ulcer	**Options**	**Answers of affected eye**
Corneal sensation?	1= Absent; 2= Reduced ; 3= Normal	
Epithelial defect	1 = Yes ; 2 = No ; 3 = Cannot measure	
	Longest dimension (mm)	
	Perpendicular measurement (mm)	

Stromal infiltrate	1 = Yes ; 2 = No ; 3 = Cannot measure	
	Longest dimension (mm)	
	Perpendicular measurement (mm)	
Evidence of suppuration	1 = Yes ; 2 = No	
Depth of deepest stromal infiltrate involvement:	1 = 0-33% 2 = 34-66% (central) 3 = 67-100%(posterior)	
Presence of corneal thinning?	1 = Yes 2 = No	
	If **yes**, Percentage (0-100%)	
Ulcer Margin?	1= Well-defined ; 2= Feathery ; 3= Not well defined	
Is there vascularisation?	1 = Yes ; 2 = No	
If yes, Up to the infiltrate	1 = Yes ; 2 = No	
Raised Margin	1 = Yes ; 2 = No	
Texture of Slough	1= Dry ; 2= Wet ; 3= None	
Immune Ring	1 = Yes ; 2 = No	
Perineural Infiltrate present?	1 = Yes ; 2 = No	
Colour of infiltrate	1= White ; 2= Yellow ; 3= NA	
Pigmentation?	1 = Yes ; 2 = No	
If yes,	1=Brown ; 2= Black	
Satellite lesions?	1 = Yes ; 2 = No	
Endothelial plaque	1 = Yes ; 2 = No	
DM Folds	1 = Yes ; 2 = No	
Descemetocoele?	1 = Yes ; 2 = No	
Perforation?	1 = Yes ; 2 = No ; 3 = Cannot measure	
If **yes**,Size & Date	Longest dimension (mm)	
	Perpendicular measurement (mm)	
Other : pls specify		

AC	**Options**	**Answers of affected eye**
KP's ?	1 = Fresh; 2 = Pigmented ; 3 = None	

Cells in AC?	1 = Yes ; 2 = No ; 3 = No view	
If **yes**, Grade:	0 = <1 cell 0.5+ = 1-5 cells 1+ = 5-15 cells 2+ = 16-25 3+ = 26-50 4+ = >50	
Flare?	1 = Yes ; 2 = No; 3 = No view	
Grade:	0 = None 1+ = Faint 2+ = Moderate (iris/lens details clear) 3+ = Marked (iris/lens hazy) 4+ = Intense (fibrin in aqueous)	
Fibrin in AC	1 = Yes ; 2 = No ; 3 = No view	
Hypopyon?	1 = Yes ; 2 = No	
If **yes**, hypopyon max. vertical height (mm)		

Probable Clinical Diagnosis:	1=Probable Fungal 2=Probable Bacterial 3=ProbableAcanthamoeba 4=ProbableViral 5=Uncertain	

Treatment started:

Name of the drug	**Dose**	**Frequency per day**	**Route (oral, e/d)**	**Start Date**	**Stop Date**

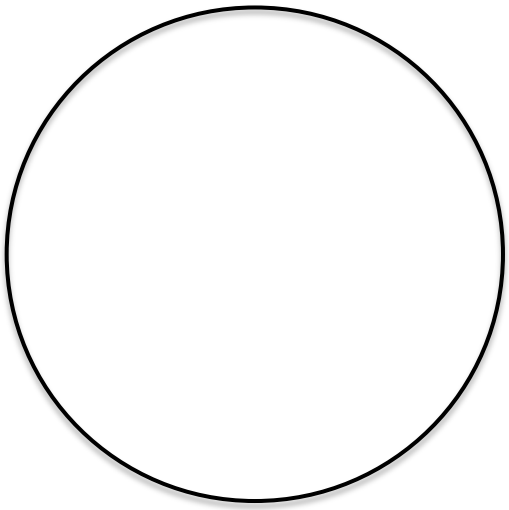
Referred to Physician?	Y/N	**Reason referred to physician:**	

DAP Study: Photo and IVCM form

M.R. No.		**Patient Name**	
Study No.		**Today's Date**	_ _ /_ _ /_ _ _ _ d d m m y y y y
Visit No.		1(Baseline)	

Photo DSC No	**Photo ID No.**
Epithelial Defect: Maximum height: _____mm Maximum width: _____mm Area : _____mm^2	Infiltrate Size: Maximum height: _____mm Maximum width: _____mm Area : _____mm^2

IVCM



Probable Diagnosis After IVCM Findings:	
1=Probable Fungal 2=Probable Bacterial 3=Probable Acanthamoeba 4=Probable Viral 5=Uncertain 6=Other Pls Specify:	