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

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Running head: Diagnostic accuracy of HRT3 IVCM in microbial keratitis

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27

Précis

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

Abstract (345 Words)

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

Design: Double-masked prospective cohort study.

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

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

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

Results: 239 patients with MK were enrolled. Fungal infection was detected in 176 (74%) and acanthamoeba in 17 (7%) by microbiology. IVCM had an overall pooled (5 graders) sensitivity of 85.7% (95% 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%). Inter-grader agreement was good: kappa=0.88 for definite fungus, kappa=0.72 for definite acanthamoeba. Intra-grader repeatability was high for both definite fungus (kappa 0.88 - 0.95) and definite acanthamoeba classification (kappa 0.63 – 0.90). IVCM images from eleven patients were considered by all five graders to have a specific organism present (ten fungus, one acanthamoeba) but were culture and light microscopy negative.

Conclusions: Laser scanning IVCM performed with experienced confocal graders has a 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 were negative.

Introduction

Severe microbial keratitis (MK) is an important cause of blindness worldwide.[1] In recent years, outbreaks of fungal and acanthamoeba keratitis 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 up to 66% of cases,[3] larger ulcers can present a diagnostic challenge as tissue destruction can 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 microbial keratitis vary widely from 40 to 73% in different settings, most likely due to 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 to culture alone,[8] but relies upon availability of trained, experienced observers who may not be present in some healthcare settings.

In vivo confocal microscopy (IVCM) is a non-invasive imaging technique that allows direct visualization of pathogens within the patient's cornea.[9] The two 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, 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 microbial keratitis *in vivo* (summarized in Labbe *et al*[9]), only two studies have prospectively assessed the diagnostic accuracy of IVCM compared to 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 to culture as the reference standard. For fungal filaments, the sensitivity was also high (94%) with a lower specificity (78%). The authors do not state whether the IVCM grader was masked to data from clinical assessment of the patient. Vaddavalli *et al* also used the Confoscan 3.0, with two 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 inter-observer agreement (kappa 0.6) was found for the two graders. Hau *et al* have previously demonstrated that the diagnostic accuracy of IVCM for the diagnosis of microbial keratitis 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 aim to determine the diagnostic accuracy of HRT3 IVCM in moderate to severe MK in South India using five experienced confocal graders (masked to microbiological diagnosis). We also assess inter and intra-grader 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 enrolment 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 as per the Standards for Reporting of Diagnostic Accuracy studies (STARD)[14] – see STARD checklist in supplementary Table 1.

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 Feb 2012 and Feb 2013 were assessed for eligibility and prospectively enrolled into the study if eligible. The inclusion criteria were: age ≥ 18 years, presence of a large corneal ulcer, defined as a stromal infiltrate ≥ 3mm in 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/examination findings were recorded in the standardized study form. We excluded any patients with a descemetocoele 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 on to the cornea for imaging), those considered to have herpetic stromal keratitis on clinical grounds (i.e. either a prior history of the disease, or 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 anaesthetized using 0.5% proparacaine eyedrops (Aurocaine, Aurolab, Madurai, India) and volume scans of the corneal ulcer were obtained using the HRT3 IVCM (Heidelberg Engineering, Germany) with Rostock Corneal Module, (63x magnification objective lens, Nikon, 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/absence of fungal filaments or acanthamoeba cysts, or if suspicious but not confidently certain then this was graded as the "possible" presence of filaments/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 on to two glass slides for light microscopy, and agar plates for culture: blood agar, (BA), potato dextrose agar (PDA) and non-nutrient agar seeded with *E. Coli* in the laboratory if acanthamoeba keratitis was clinically suspected. Standard microbiological methods were followed to detect any pathogen.[15] In brief, slides were stained with 10% potassium hydroxide or gram stain or giemsa to aid visualization of fungal filaments, bacteria or acanthamoeba cysts respectively; agar plates were incubated at 37^{o}C for 2 days for BA, or at 27^{o}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 two solid media, or, b) semi-confluent growth at the site of inoculation in one 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 & 2; grader 2 with an additional 2 years of general IVCM experience), 3.5 years (grader 3), and 2 years (graders 4 & 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 database. To measure intra-grader 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 and/or presence of acanthamoeba cysts on light microscopy; this approach has previously been shown to increase diagnostic accuracy for acanthamoeba detection, compared to 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 a limited clinical history available to them on the microbiology test request form.

Statistical methods

All statistical analyses were performed in Stata 12.1 (StataCorp, Texas, USA). Sample size was estimated as n=200 based on a fungal keratitis prevalence estimate of 50%, aiming for sensitivity of 85%, and with marginal error of 7%, as per 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 squared 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 (PPV) and negative predictive value (NPV) 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 two measures (*metandi* and *midas* commands in Stata).[20-22] This is likely to be a conservative estimate since it accounts for the various level 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 kappa score to calculate intra-grader agreement (to assess reproducibility). A kappa score was also calculated for inter-grader agreement (to assess reliability) for cases graded with certainty as "definite fungus/acanthamoeba" or "no organism seen". Kappa scores were interpreted as follows: ≤0.20 "no agreement"; 0.21-0.39 "minimal agreement"; 0.40-0.59 "weak agreement"; 0.60-0.79 "moderate agreement"; 0.80-0.90 "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 for STARD patient flow diagram. A total of 3163 volume scans were obtained with a mean 13 volume scans per patient (range 3-42). 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/light microscopy.

Socio-demographic features of the final participants are shown in Table 1. Compared to 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. The majority of 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 were 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 1b shows an example of fungal filaments as seen in IVCM images of a culture positive fungal ulcer. Overall, all five graders were able to definitely detect fungal filaments in the IVCM images with a pooled sensitivity of 85.7% (95% 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 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 four 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%). Earlier presentation with shorter symptom duration (≤4 days) had the highest pooled sensitivity for all 5 graders of 95% (95% CI: 88 - 98%) but lowest pooled specificity of 53% (95% CI: 39% - 66%). As symptom duration increased to longer than 10 days, the pooled sensitivity reduced to 72% (95% CI: 64% - 78%), with concomitant increase in sensitivity to 91% (95% CI: 84% – 95%), as shown in Table 5.

There was a strong inter-grader agreement between all five masked graders' scores for definite fungus, with a kappa score of 0.88 ($p<0.0001$). Kappa scores for intra-grader 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 three culture-positive *Nocardia sp*. cases were classed as not having filamentous structures by 4 out of the 5 graders.

IVCM "false positives" or "false negatives" for fungus

Ten patients were microbiologically negative for fungus but four or more 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. Nine of these ten ulcers were noted to be deep with extension into the posterior third of the cornea on slit lamp examination and/or IVCM imaging.

Conversely, nine 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 up to 21 days after presentation, fungal filaments were still not detected in five patients and the remaining four 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 for this patient.

IVCM Detection of Acanthamoeba Cysts

For definite detection of acanthamoeba cysts, all five 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 four graders masked to clinical features had a very similar pooled

sensitivity of 88.5% (95% CI 73.0% - 95.6%) and pooled specificity of 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) was 82% (95% CI 34 - 98%) and 98% (95% CI 95 - 99%) respectively. This high sensitivity and specificity was maintained in ulcers with longer symptom duration beyond 30 days (see Table 5).

For all 5 graders, there was a moderate inter-grader agreement with kappa score 0.72 ($p<0.0001$). Kappa scores for intra-grader 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 one IVCM "false positive" case, culture and light microscopy 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 cyst-like 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, two of the five graders classified the images for this ulcer as "possible acanthamoeba".

"Possible" fungus or acanthamoeba on IVCM

Seventy-one ulcers in total were classified as "possible" fungus present by any grader, with agreement from 3 or more 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/light microscopy positive for *Acanthamoeba sp*. (n=3), *Nocardia sp*. (n=2) or *Streptococcus 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 re-grading, up to 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 reference standard positive for fungus (n=29/34). Of the images initially graded as "possible acanthamoeba", 9% (n=8/88) were shifted to the "definite acanthamoeba" grade at re-grading, 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). Six of these images 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 one 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 and acanthamoeba keratitis in particular can lead to significant visual loss, and even loss of the eye.[24-26] IVCM is a non-invasive 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 fungal keratitis, but for acanthamoeba keratitis there was sufficient evidence to support the use of IVCM as the sole diagnostic test.[27] Since then, two prospective studies using the Confoscan IVCM have found a high sensitivity and specificity for the detection of fungal filaments and acanthamoeba cysts.[11,12] In this report, we provide for the first time evidence of a 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 two studies. Use of a multi-grader 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 to the study by Vaddavalli *et al*. We were only able to study a small

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

We have found that experienced IVCM graders were able to detect fungi or acanthamoeba in 94.8% of all culture and/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 five of our nine IVCM "false negative" fungal ulcers. A small number of patients were only able to tolerate IVCM imaging for 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.[28] 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 whole 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 reducing image contrast as with surface plaques, or difficulty in identifying acanthamoeba cysts in the presence of a large number of white cells since they both have similar morphology).

We have found that IVCM graders were able to detect a pathogen in 11 culture and light microscopy negative ulcers. The IVCM images in these ulcers had classical features of fungal hyphae or acanthamoeba cysts and so we feel these represent true cases of disease. In the majority of 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 on light microscopy. In such cases, IVCM is an invaluable tool to rapidly detect fungal filaments in the deep stroma and allows the correct antimicrobial treatment to be commenced without the need for invasive corneal biopsy to identify the pathogen.[29] 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.[30] Only 1 grader out of 5 classified images from Nocardia keratitis as containing fungal filaments in this study. Since *Nocardia sp*. filaments are thinner in diameter than filamentous fungi (<1.5 microns versus 3-6 microns resp.),[31] they can be more difficult to detect on IVCM particularly in the presence of significant stromal oedema or inflammation as in moderate to severe keratitis, but were readily detected microbiologically in our study.

In the clinical setting, an uncertain IVCM test result can cause concern with regards to which antimicrobial therapy to commence. On further analysis of all images graded as showing "possible" presence of a pathogen, 75% of those graded as "possible fungus" were appropriately classified when compared with the reference standard, but less than 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

443 imaging to reach a definite diagnosis for acanthamoebal infection in particular, rather
444 than using one diagnostic tool alone, as also found by others.[18]
445
446 There was an apparent improvement in the certainty of diagnosis on re-grading
447 images. This learning effect was also detected by Hau *et al*, who found that the
448 specificity improved for all graders upon IVCM MK image re-grading at a later
449 date.[13] They also found that as the level of IVCM experience of the grader
450 increased,[13] the diagnostic accuracy for detection of MK also improved, thus
451 indicating the importance of training in IVCM image recognition for all new graders.
452 The IVCM grader may also benefit from having access to a clinical image of the
453 ulcer,[18] since our grader with access to clinical feature information had a higher
454 sensitivity for fungal detection.
455
456 In this study, although the graders were from a variety of backgrounds (ophthalmic
457 nurses, optometrists and ophthalmologists) and levels of experience, they had a high
458 inter-grader agreement for pathogen detection. We found higher kappa scores for
459 inter-grader agreement than Vaddavalli *et al*,[12] which may be due to the higher
460 resolution of the HRT3 imaging system allowing for higher definition images of the
461 pathogen, as well as the training/experience of our confocal graders with this high
462 resolution imaging system. Intra-observer agreement was in our study was also high,
463 and was better for fungal detection with the best agreement in the most experienced
464 observer.
465
466 Limitations of this study include the dominance of filamentary fungal keratitis, and
467 the relatively low proportion of bacterial infections. We were unable to study confocal

appearances of candida keratitis, which is more common in more temperate climates. We only studied 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 and acanthamoeba keratitis, in low and middle income countries in tropical regions; however, delay in treatment may result in a greater cost in the long term due to 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. Firstly, we used a microbiology service that is particularly optimized for ocular microbiology. Secondly, culture could be initiated with very little delay after sample collection since the laboratory is situated next to the Cornea Clinic at Aravind Eye Hospital. Thirdly, the standard practice is to use a kimura spatula, which we also believe gives a more ample sample than using a needle, 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

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

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

Figure 2: *In vivo* confocal microscopy images of six culture and light microscopy negative ulcers in which graders detected fungal filaments (A-E) or acanthamobea 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.

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

Table Legends

Table 1: Demographic data and clinical features of study participants

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

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

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

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

Supplementary Table: STARD Checklist

Table 1: Demographic data and clinical features of study participants

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

[a] Mixed infections included (culture positive for bacteria but microscopy positive for fungus, n=2
[b] For prior drug use, n=209 (data not available for 30 patients)

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

Culture positives (n=182)	**N**	**%**
Acanthamoeba	17	7.1%
Fungi	144	60.3%
Bacteria	19	9.6%
Mixed: Culture +ve for bacteria, microscopy +ve for fungi	2	0.8%
Culture negatives (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%**

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 positives	*Streptococcus pneumoniae*	10	47.6%
	Streptococcus viridans	3	14.3%
	Staphylococcus epidermidis	2	9.5%
	Nocardia sp.	3	14.3%
Bacteria: Gram negatives	*Pseudomonas aeruginosa*	2	9.5%
	Aeromonas sp.	1	4.8%

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

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

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

* The total no. of patients classified as having "Possible fungus" by each grader and therefore excluded from this analysis are as follows: Grader 1 (n=21), Grader 2 (n=23), Grader 3 (n=49), Grader 4 (n=16), Grader 5 (n=1)

[a] Grader 5 was unmasked to ulcer clinical features.

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

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

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

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

[a] Grader 5 was unmasked to ulcer clinical features.

Table 5 revised

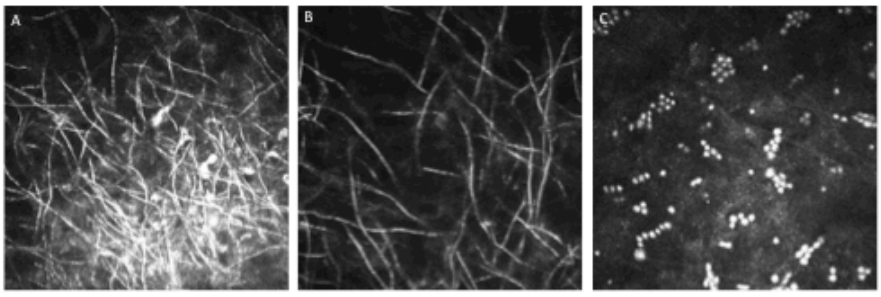
Table 5: Pooled Sensitivity and Specificity for all 5 graders by Symptom Duration (Split by Quartile for Fungi, by Median for Acanthamoeba)

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

Supplemental Table - Online Only

SUPPLEMENTARY TABLE: STARD CHECKLIST

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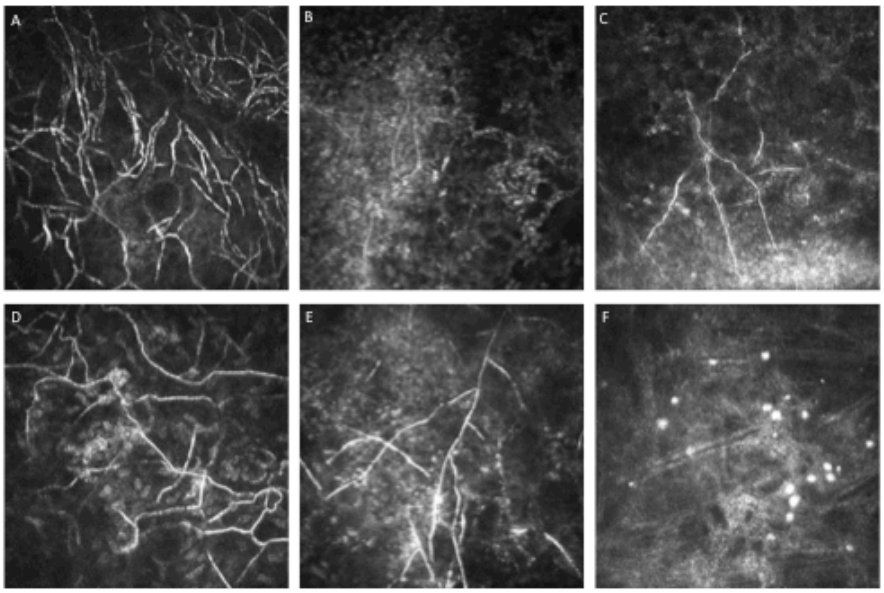
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Supplemental Figure - Online Only

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