Sensitivity and Specificity of Laser-Scanning In Vivo Confocal Microscopy for Filamentous Fungal Keratitis: Role of Observer Experience

AHMAD KHEIRKHAN, ZEBA A. SYED, VANNAURAT SATITPITAKUL, SUNALI GOYAL, RODRIGO MÜLLER, ELMER Y. TU, AND REZA DANA

- PURPOSE: To determine sensitivity and specificity of laser-scanning in vivo confocal microscopy (LS-IVCM) for detection of filamentous fungi in patients with microbial keratitis and to evaluate the effect of observer’s imaging experience on these parameters.
- DESIGN: Retrospective reliability study.
- METHODS: This study included 21 patients with filamentous fungal keratitis and 24 patients with bacterial keratitis (as controls). The etiology of infection was confirmed based on the response to specific therapy regardless of culture results. All patients had undergone full-thickness corneal imaging by a LS-IVCM (Heidelberg Retina Tomograph 3 with Rostock Cornea Module; Heidelberg Engineering, Heidelberg, Germany). The images were evaluated for the presence of fungal filaments by 2 experienced observers and 2 inexperienced observers. All observers were masked to the clinical and microbiologic data.
- RESULTS: The mean number of images obtained per eye was 917 ± 353. The average sensitivity of LS-IVCM for detecting fungal filaments was 71.4% ± 0% for the experienced observers and 42.9% ± 6.7% for the inexperienced observers. The average specificity was 89.6% ± 3.0% and 87.5% ± 17.7% for these 2 groups of observers, respectively. Although there was a good agreement between the 2 experienced observers (κ = 0.77), the inexperienced observers showed only a moderate interobserver agreement (κ = 0.51). The LS-IVCM sensitivity was higher in patients with fungal infections who had positive culture or longer duration of the disease.
- CONCLUSIONS: Although LS-IVCM has a high specificity for diagnosing filamentous fungal keratitis, its sensitivity is moderate and highly dependent on the level of the observer’s experience and training with this imaging modality. (Am J Ophthalmol 2017;179:81–89. © 2017 Elsevier Inc. All rights reserved.)

Fungal keratitis is a potentially blinding disease that can cause significant damage to the cornea. Whereas fungi are an uncommon cause of microbial keratitis in temperate climates, they are very common in tropical environments, where fungal keratitis accounts for 30%–62% of corneal infections.1–3 Fungal corneal infections are mostly caused by filamentous fungi, such as Aspergillus and Fusarium species, and less commonly by yeasts such as Candida species.1–3 Clinical manifestations of fungal keratitis are often nonspecific, which makes it difficult to differentiate these infections from other forms of microbial keratitis.4–6 Early diagnosis of fungal corneal infections is the most important determinant of their prognosis.1–3 However, diagnosis of these infections is often challenging because they can present as chronic infiltration or even with an intact epithelium.

The gold standard for diagnosing fungal keratitis is culture results from corneal scrapings; however, the slow growth of filamentous fungi, which may take weeks, remains an impediment.3,7 Furthermore, the sensitivity of fungal culture is low, with less than half of the cases showing a positive culture.8–10 This problem is further compounded by their often deep location in the stroma, which makes the yield from scrapings low. Although histopathologic evaluation of corneal biopsy specimens as well as polymerase chain reaction (PCR) techniques have been used for diagnosis of fungal keratitis, these are invasive or not available in many laboratories.11,12 These factors may lead to a delay in diagnosis, with the potential for a significant visual loss.13 Therefore, it is critical to have a diagnostic modality that is easy to use, sensitive, and specific, and that can provide rapid diagnosis of fungal keratitis.

In vivo confocal microscopy (IVCM) is a noninvasive imaging modality that allows real-time imaging of corneal structures at a cellular level.14–16 Two most commonly used types of corneal IVCM include slit-scanning (ConfoScan; Nidek Technologies) and laser-scanning (Heidelberg Retina Tomograph with Rostock Corneal Module;
Heidelberg Engineering) technologies, with the latter having a better axial resolution.13 Numerous studies have demonstrated the utility of IVCM, both slit-scanning and laser-scanning, for rapid detection of filamentous fungal elements in corneal infections.17–23 However, there are limited data on the sensitivity and specificity of laser-scanning IVCM (LS-IVCM) for detection of fungal filaments in these infections.24,25 Furthermore, in the few published studies, only patients with positive fungal smear and/or culture have been included; this might have resulted in an overestimation of the sensitivity of IVCM for detection of fungal elements. On the other hand, in these studies IVCM images have been analyzed only by experienced observers, with different levels of experience.24,25 Therefore, as many cornea clinicians do not have sufficient specialized training on interpretation of IVCM images, it is important to know the accuracy of the image interpretation by these relatively inexperienced clinicians as well.

The aim of this study is to report the sensitivity and specificity of LS-IVCM for detection of fungal filaments in patients with proven fungal keratitis regardless of culture results. Furthermore, we evaluated the effects of observer experience on these parameters.

METHODS

TO IDENTIFY PATIENTS WITH FUNGAL KERATITIS AS WELL as bacterial keratitis (as the control group), we retrospectively reviewed clinical records and images of patients with infectious keratitis who had undergone LS-IVCM imaging at Massachusetts Eye and Ear Infirmary, Boston, Massachusetts. Protocol of the study was approved by the Human Studies Committee of Massachusetts Eye and Ear Infirmary from May 2008 through July 2015. The electronic medical records of all these patients were reviewed for results of microbial culture, treatments regimens, and clinical response to the treatment. Out of these patients, 21 with fungal keratitis met the inclusion and exclusion criteria. For the control group, 24 patients with bacterial keratitis were also randomly selected from the same patients’ pool.

• PARTICIPANTS: Patients with infectious keratitis had received LS-IVCM imaging if, regardless of the ulcer size, their clinical presentations had not been typical for bacterial keratitis. All patients had a complete anterior segment assessment, including slit-lamp examination, and microbiologic evaluation with smear and culture of corneal scrapings. Diagnosis of the underlying etiologic organism was confirmed in each patient based on the response to specific therapy, regardless of culture results. For this, the response was defined as resolution of infiltration or significant reduction of the size of corneal infiltrate within 7 days of antibacterial treatment (fortified antibiotics) for bacterial keratitis or within 2 weeks of antifungal therapy for fungal keratitis. For example, even with a negative culture result, if the patient had responded to the antifungal therapy alone, which had been started owing to a clinical suspicion for fungal corneal infection, the patient was categorized as having fungal keratitis. Patients who had received any combination of antibacterial, antifungal, antiviral, and antiacanthamoebal agents were excluded. Furthermore, those with yeast keratitis were also excluded from the study.

More than 660 patients had undergone LS-IVCM imaging for infectious keratitis at Massachusetts Eye and Ear Infirmary from May 2008 through July 2015. The electronic medical records of all these patients were reviewed for results of microbial culture, treatments regimens, and clinical response to the treatment. Out of these patients, 21 with fungal keratitis met the inclusion and exclusion criteria. For the control group, 24 patients with bacterial keratitis were also randomly selected from the same patients’ pool.

• IMAGE ANALYSIS: All images of the first imaging visit of each patient were exported as JPG files without any identifiable data. Four observers who were masked to the clinical data and diagnosis of patients interpreted the LS-IVCM images regarding the presence or absence of filamentous fungal elements. These observers included 2 cornea specialists with ≥3 years of experience with LS-IVCM imaging (so-called “experienced observers”)
RESULTS

THIS STUDY INCLUDED 45 EYES OF 45 PATIENTS (22 WOMEN and 23 men) with mean age of 51.3 ± 17.3 years (range, 18–88 years). They consisted of 21 patients with filamentous fungal keratitis and 24 patients with bacterial keratitis (the control group). Duration of the disease ranged from 1 day to 2 months, and 23 (51.1%) were contact lens wearers. The culture results were positive in 11 patients (52.4%) with fungal keratitis (showing filamentous fungi) and in 16 patients (66.7%) with bacterial keratitis (excluding 4 cultures that were positive for coagulase-negative staphylococci).

Imaging with LS-IVCM had been performed uneventfully for all patients. The mean number of images per eye analyzed by each observer was 917 ± 353 (range, 300–1955 images).

Of 21 patients with filamentous fungal keratitis, LS-IVCM images were considered positive by all 4 observers in 8 cases showing typical highly reflective lines with numerous, interlocking branchings (Figure 1). In 5 patients with fungal keratitis, none of the observers detected any fungal filaments in LS-IVCM images. In 6 patients with fungal keratitis, although both experienced observers detected fungal elements, such filaments were not recognized by 1 (n = 3 patients) or both (n = 3 patients) inexperienced observers (Figure 2). In 2 other patients with fungal keratitis, only 1 experienced observer detected the fungi, whereas other observers could not find such elements (Figure 3). On the other hand, of 24 patients with bacterial keratitis, fungal elements were erroneously detected in 2 patients by 2 observers and in 7 patients by 1 observer (Figure 4).

The sensitivity, specificity, positive predictive value, negative predictive value, false-positive rate, and false-negative rate of LS-IVCM for detecting filamentous fungal elements for both experienced and inexperienced observers are shown in Table 1. The average sensitivity of LS-IVCM for detecting fungal elements was 71.4% ± 0% for experienced observers and 42.9% ± 6.7% for inexperienced observers. The average specificity was 89.6% ± 3.0% and 87.5% ± 17.7% for these 2 groups of observers, respectively. The average positive and negative predictive values were 85.8% ± 3.5% and 78.2% ± 0.6% for experienced observers and 78.6% ± 30.3% and 63.4% ± 7.4% for inexperienced observers, respectively. The average false-positive and false-negative rates were 10.4% ± 3.0% and 28.6% ± 0% and for the experienced group and 12.5% ± 17.7% and 57.2% ± 6.7% for the inexperienced group, respectively.

For experienced observers, the average sensitivity of LS-IVCM for detecting filamentous fungal keratitis was 81.8% in those who had positive fungal cultures and 60% in patients with negative fungal cultures. On the other hand, duration of the disease was shorter in patients with fungal keratitis whose LS-IVCM images were interpreted as negative by
FIGURE 1. Examples of laser-scanning in vivo confocal microscopy images of patients with fungal keratitis that all observers, whether experienced or inexperienced, considered as positive for fungal filaments. Typical highly reflective lines with numerous, interlocking branchings are seen in all images.
both experienced observers (8.1 ± 5.0 days, n = 5 patients) compared with those whose images were interpreted as positive by at least 1 experienced observer (18.2 ± 12.7 days, n = 16 patients), with the difference being statistically significant (P = .04).

Interobserver agreements for both groups of observers are shown in Tables 2 and 3. For experienced observers, there was a good agreement between the 2 observers, with \( \kappa = 0.77, P < .001 \). For inexperienced observers, there was only a moderate agreement between the 2 observers, with \( \kappa = 0.51, P < .001 \).

For experienced observers, the repeat image analysis resulted in very high intraobserver agreements for both observer 1 (\( \kappa = 0.86, P < .001 \)) and observer 2 (\( \kappa = 0.91, P < .001 \)). For the repeat analysis, the average sensitivity and specificity were 71.4% ± 0% and 91.7% ± 5.9%, the average positive and negative predictive values were 88.6% ± 7.4% and 78.6% ± 1.1%, and the average false-positive and false-negative rates were 8.3% ± 5.9% and 28.6% ± 0%, respectively.

**DISCUSSION**

ALTHOUGH BACTERIA CANNOT BE DETECTED BY LS-IVCM owing to their small size, filamentous fungi can be seen as highly reflective linear structures with a diameter of 3–10 \( \mu \text{m} \) that have irregular, numerous branchings with an interlocking pattern.\(^{17-19} \) For detection of these fungal filaments, herein we noted an average sensitivity of 71% for experienced observers and 43% for inexperienced observers. However, the specificity was 75% or more for all observers, whether experienced or inexperienced.

Using LS-IVCM, Hau and associates reported correct diagnosis rates of only 8.3%–41.2% for fungal keratitis by 4 different observers with various levels of experience.\(^{24} \) Using the LS-IVCM, Chidambaram and associates also demonstrated that 4 experienced observers had sensitivity of 79.1%–86.8% and specificity of 73.7%–85.9% for detection of fungal elements.\(^{25} \) Such a higher sensitivity (a lower false-negative rate) can be attributed to the fact that they

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FIGURE 2. Examples of laser-scanning in vivo confocal microscopy images of patients with fungal keratitis in which although both experienced observers detected fungal elements, such elements were not recognized by 1 or both inexperienced observers. Although highly reflective branching lines are seen, they are less obvious compared with Figure 1. As these were present in only a few frames among hundreds of images for each patient, they might have been missed.
included only patients with moderate to severe keratitis (eg, infiltrate diameter ≥ 3 mm) who had positive culture or light microscopy results. In addition, they excluded those whose images were not definite for fungal infection, which would increase the diagnostic sensitivity. On the other hand, using slit-scanning IVCM in patients with smear/culture-positive fungal keratitis, sensitivity of 89%–94% and specificity of 78%–93% have also been reported in other studies.27,28

Inclusion of only those with positive microbiologic or histologic data, as performed in previous studies, differs from what is seen in daily practice, in which only half of patients with fungal keratitis show positive cultures.8–10 In fact, this inclusion may cause a significant overestimation of the IVCM sensitivity for detection of fungal keratitis, as those with culture-positive fungal keratitis may have a higher load of fungal elements in the cornea. This is confirmed by our finding that the average sensitivity for experienced observers was higher in patients with positive fungal cultures (82%) than in those with negative cultures (60%). That is why we also included patients with negative cultures who were clinically considered as fungal keratitis owing to their response to antifungal treatment. In fact, it is this group of patients (with negative or unknown culture results) who present diagnostic challenges, and for them IVCM is most useful.

As reported previously,24,25 we observed a notable effect of the observer’s imaging experience on the sensitivity of LS-IVCM for detection of fungal filaments in corneal infections. To reduce the gap between experienced and inexperienced observers in this study, all observers were asked to study the published literature before starting the analysis. Despite this, inexperienced observers had false-negative rates (57%) almost twice those of experienced observers (29%). On the other hand, although the specificity was around 90% for experienced observers, it ranged from 75% to 100% for inexperienced observers. The inexperienced observer with a specificity of 100%, and thus false-positive rate of 0%, showed a low sensitivity. Such variation in interpretation of results owing to the observer’s imaging experience is also reflected in the interobserver agreement. Although experienced observers had good or

FIGURE 3. Examples of laser-scanning in vivo confocal microscopy images of patients with fungal keratitis in which only 1 experienced observer detected the fungi, whereas other observers could not find such elements. These highly reflective lines, which do not show the typical pattern seen in Figure 1, could easily be missed.
very good interobserver and intraobserver agreements, as described by others, there was only a moderate agreement between inexperienced observers. Such findings signify the importance of previous experience with IVCM in interpreting images, and this cannot be substituted by just reviewing the literature. This is especially true for eye care centers in which fungal keratitis is not a common occurrence.

In addition to the observer experience, the sensitivity and specificity of IVCM for diagnosis of fungal keratitis could be significantly affected by inherent characteristics of this imaging modality. Despite high resolution, IVCM suffers from the limitation of a small field size, which is only 0.15% of the total corneal area. Thus, more than 100,000 non-overlapping images are needed to evaluate the full-thickness of the whole cornea. To make the process clinically feasible, in this study we took Sequence Scans

FIGURE 4. Examples of laser-scanning in vivo confocal microscopy images of patients with bacterial keratitis in which 1 or 2 observers erroneously detected fungal elements (false-positive).

### TABLE 1. Sensitivity, Specificity, False-Positive Rate, and False-Negative Rate of Laser-Scanning In Vivo Confocal Microscopy for Detecting Filamentous Fungal Elements for Both Experienced and Inexperienced Observers

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Experienced Observers</th>
<th>Inexperienced Observers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observer 1</td>
<td>Observer 2</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>71.4%</td>
<td>71.4%</td>
</tr>
<tr>
<td>Specificity</td>
<td>91.7%</td>
<td>87.5%</td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>88.2%</td>
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<tr>
<td>Negative predictive value</td>
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</tr>
<tr>
<td>False-positive rate</td>
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<td>12.5%</td>
</tr>
<tr>
<td>False-negative rate</td>
<td>28.6%</td>
<td>28.6%</td>
</tr>
</tbody>
</table>

### TABLE 2. Interobserver Agreement for Detection of Filamentous Fungal Elements in Laser-Scanning In Vivo Confocal Microscopy Images in the Group of Experienced Observers

<table>
<thead>
<tr>
<th>Observer 2</th>
<th>Positive Fungal Elements</th>
<th>Negative Fungal Elements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observer 1</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>25</td>
</tr>
</tbody>
</table>

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from the full-thickness of the cornea in and around the ulcer with an average image number of more than 900. This is different from a previous study in which only the most typical image of each patient was used to evaluate the sensitivity and specificity of LS-IVCM for microbial keratitis.24 Such a high number of images in our study still may not be enough to evaluate the presence of organisms in the cornea if their number is low, resulting in a false-negative result. In addition, a false-negative result may be attributable to the technical difficulties in acquiring adequate numbers of IVCM images, such as poor patient cooperation or presence of superficial plaques.25 Furthermore, the presence of a large number of inflammatory cells, necrosis, or opacity in the stroma may also obscure the organisms, leading to a false-negative result. On the other hand, filament-like structures such as nerves, disorganized collagen fibers, and dendritic cell processes may be mistaken as fungal filaments, resulting in a false-positive interpretation when the typical morphology of filaments is not seen.29–31 Evaluation of the captured videos, in addition to the still images, may be more helpful in differentiating these structures.

To prevent bias, in this study we masked the observers to the clinical data. However, in the clinical setting, IVCM should not be used as a stand-alone test, as it has been shown that IVCM graders demonstrate an increased diagnostic accuracy when they have access to the clinical data.23 On the other hand, we noted a higher positive rate for fungal elements in those with longer duration of the disease. This direct correlation may be owing to a higher load of the organism in those with a protracted course. Conversely, Chidambaram and associates reported a higher sensitivity in patients with shorter disease duration.25

A limitation of our study is the retrospective design. In addition, we excluded those with Candida keratitis, because the morphologic features of yeasts have not been well defined in IVCM.20,32 On the other hand, even though different morphologies have been described for different species of filamentous fungi,18,19 such distinction was not performed in our study as this may not be easy in IVCM images. This difficulty has been confirmed by a recent study.33 In addition, it has been shown that fungal keratitis may occasionally respond to antibiotics such as tobramycin or moxifloxacin34,35; therefore, there is a slim possibility that some of the culture-negative patients who had responded to antibiotic therapy had fungal keratitis.

In conclusion, LS-IVCM is a useful imaging modality for rapid, noninvasive detection of filamentous fungal elements in corneal infections, particularly in patients with deep infiltrates that are not accessible to scrapings or those on antifungal therapy who may have a low microbiologic yield.25,28,36 However, a significant experience with a steep learning curve28 is required to achieve a high sensitivity, which would be better than microbiologic studies.20,37 If available, IVCM can be used as an initial step for diagnosing fungal keratitis.

### REFERENCES
