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Filamentous Fungal Keratitis in Taiwan: Based on Molecular Diagnosis

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Purpose: To analyze the epidemiological pattern, demographics, risk factors, and treatment outcomes of filamentous fungal keratitis at a tertiary hospital in Taiwan.

Methods: We recruited 65 patients (65 eyes) with culture-proven filamentous fungal keratitis who received diagnosis and treatment at Chang Gung Memorial Hospital between 2015 and 2018. All isolates were examined through conventional morphological identification and subjected to molecular identification with internal transcribed spacer sequencing. Data on patient demographics, predisposing factors, and treatment outcomes were collected.

Results: In total, filamentous fungi belonged to 16 genera were identified. *Fusarium* spp. (29 cases [44.6%]) was the most commonly isolated organism overall, followed by *Colletotrichum* spp. and *Purpureocillium linacinum* (seven cases [10.8% for each]), and *Aspergillus* spp. (six cases [9.2%]). Some fungi that have not been regarded as human pathogens were also identified, such as *Paracremonium* and *Phellinum*. Among 52 (80%) patients with predisposing factors, 30 (46.2%) had trauma. The ulcers of 33 (50.8%) patients resolved with medical treatment only. Additionally, six patients (9.2%) had corneal perforation, and nine patients (13.9%) required therapeutic/destructive surgical interventions including therapeutic penetrating keratoplasty (seven patients) or evisceration (two patients). Only 16 patients (24.6%) had final visual acuity of 20/40 or better.

Conclusions: Through molecular diagnosis, a high diversity of fungal pathogens was revealed along with an increasing incidence of *Colletotrichum spp.* and *Purpureocillium spp.* in Taiwan. The most common risk factor for filamentous fungal keratitis was trauma. The visual outcomes were guarded.

Translational Relevance: The molecular diagnosis provides insight into accurate identification, which affects the epidemiology and diversity of pathogens of filamentous fungal keratitis.

Introduction

Fungal keratitis is a serious cornea disease that can lead to reduced vision or even blindness.[1,2] In tropical and subtropical regions, filamentous fungi account for more than 50% of all microbial keratitis cases.[3] During the past decades, increasing incidence of filamentous fungal infections was noted globally. Although *Fusarium spp.* and *Aspergillus spp.* are the most frequent causative agents, more than 70 filamentous fungal species have been reported as pathogens.[4] The epidemiological pattern of filamentous fungal keratitis varies throughout different countries, geographic regions, and even among regions of the same country.[4] Thus local epidemiological studies provide valuable information for clinical practice.

Early diagnosis of filamentous fungal keratitis and prompt application of antifungal agents are essential to avoid vision-threatening outcomes. Although

some predisposing factors and clinical features may raise a physician's awareness of possible filamentous fungal infection,[5] laboratory investigations remain the cornerstone of diagnosis. Although culture-based methods are the most frequently selected diagnostic tools, they are labor and time intensive.[4] In addition, morphological identification requires a solid background knowledge of mycology to identify some uncommon fungi correctly.[6] By contrast, molecular-based methods provide accurate, consistent, and timely diagnoses. They enable accurate identification even with small amounts of target DNA or dead fungal elements that cannot grow,[7] and they provide crucial information not only for promoting our understanding of the spectrum of etiologic agents but also for gaining essential prognostic and therapeutic information for patient care.

To date, epidemiologic studies on microbial keratitis in Taiwan have mainly focused on bacterial rather than fungal infections.[8,9] In our hospital, we have used sequence data analysis of the internal transcribed spacer (ITS) region in addition to conventional morphologic classification for the diagnosis of filamentous fungal keratitis since 2015. Herein, we review the clinical features, laboratory findings, and treatment outcomes of filamentous fungal keratitis over a four-year period (year 2015–2018) in our hospital, a tertiary medical center in Taiwan.

Methods

Ethics

This study was in accordance with the Declaration of Helsinki and approved by the Institutional Research Ethics Board at Chang Gung Memorial Hospital, Taiwan, which granted a waiver of consent because patient anonymity was maintained by the data source.

Patients and Data Collection

We searched the computer database of the microbiology laboratory in our hospital and reviewed the corresponding medical records to identify patients with culture-proven filamentous fungal keratitis treated between January 1, 2015, and December 31, 2018. Corneal scrapings obtained from patients with presumed infectious keratitis were sent for smear and culture examinations to detect bacteria, mycobacteria, and fungi using standard microbiological culture techniques, including the use of blood and chocolate agar, modified Sabouraud agar, Lowenstein–Jensen agar slants, and thioglycolate broth. Fungal cultures were defined as positive if any fungal growth on two media, fungal elements seen in smears and fungal growth on one medium or confluent fungal growth on one medium. All fungal isolates were identified by their structure, then all filamentous fungal isolates were subjected to molecular identification. Data on patient demographics, medical and ocular history, signs and symptoms, predisposing factors, presenting and final visual acuity, treatment, and follow-up duration were collected.

We defined an ulcer as central if it encroached within 2 mm of fixation, peripheral if it involved a zone within 2 mm from the limbus, and paracentral if it was located between the central and peripheral zones. Corneal ulcers were defined as small (<2 mm), medium (2–6 mm), and large (>6 mm). Predisposing risk factors were classified into 6 subgroups: trauma, the wearing of contact lenses, topical steroid use, preexisting ocular disorders, systemic disorders, and recent ocular surgery. Preexisting ocular disorders was defined as any disease that could damage the corneal epithelium. Systemic disorders included systemic disease with ocular involvement. Recent ocular surgery was defined as surgery that occurred within three months of the onset of fungal keratitis.

If data were available, visual outcome was recorded as best corrected visual acuity (BCVA) using Snellen charts. The final vision was defined as BCVA at last follow-up, which was consistent in two consecutive visits. For analysis, Snellen visual acuity (VA) was converted to logarithm of the minimum angle of resolution VA (logMAR). The following logMAR values were assigned for nonnumeric VAs: counting fingers, 2 logMAR; hand movement, 2.3 logMAR; light perception, 2.7 logMAR; and no light perception, 3.0 logMAR, as suggested by Schulze-Bonsel et al.[10] and Bach et al.[11]

Linear stepwise regression was used to determine factors associated with final visual outcome. Statistical significance was defined as $P < .05$. SPSS version 25 (IBM, SPSS Statistics, New York, NY, USA) was used for statistical analysis.

DNA Extraction, Amplification, and Sequencing

The fungal isolates were sub-cultured on potato dextrose agar for purification. Then, part of the fungal mycelia was collected and placed in a plastic vial, to which 0.13 g of metal beads and 800 µL of lysis buffer were added. The vial was then transferred to a cell disruptor (Mini-BeadBeater 16; BioSpec, Bartlesville, OK, USA) to break down the fungal cell wall. Buffer

fluid containing fungal fragments was transferred to the DNA extraction kit, and genomic DNA was extracted with a Smart LabAssist (TANBead, Taoyuan City, Taiwan) automatic DNA extraction system. The primers ITS1 and ITS4 were used to amplify the ITS regions and 5.8S gene of ribosomal DNA in accordance with previously published protocols. An ABI Prism model 3730 ×l DNA Analyzer (Applied Biosystems, Foster City, CA., USA) was used for DNA sequencing.

Results

Microbiological Results

Over the four-year study period, 65 filamentous fungal keratitis cultures were classified using ITS as well as conventional morphological identification. Fifty-three isolates had the same diagnosis by both methods, but ten had different diagnosis (marked in Table 1); two could not be identified by culture. According to ITS-based identification presented in Table 1, *Fusarium* spp. (29 cases, 44.6%) was the most commonly isolated organism, followed by *Colletotrichum* spp. (seven cases, 10.8%) and *Purpureocillium lilacinum* (formerly *Paecilomyces lilacinum*) (seven cases, 10.8%), and *Aspergillus* spp. (six cases, 9.2%), *Curvularia* spp. (four cases. 6.2%), and *Scedosporium* spp. (two cases, 3.1%). In 29 *Fusarium* isolates, 23 belonged to *Fusarium solani* species complex (SC), three were *Fusarium oxysporum* SC, two were *Fusarium dimerum* SC, and one was *Fusarium fujikuroi* SC. In seven *Colletotrichum* isolates, five were *Colletotrichum gloeosporioides* SC, two were *Colletotrichum truncatum* SC.

Demographics, Clinical Features, and Risk Factors

As shown in Table 2, of the 65 patients, men (49, 75.4%) outnumbered women (16, 24.2%) by approximately three to one. Their ages ranged from 19 to 86 years (mean 57.0 ± 18.0 years). Thirty-four eyes (51.5%) of corneal ulcers were medium-sized, and 40 eyes (61.8%) were in the paracentral area. Hypopyon was present in 24 eyes (36.4%).

In Table 3, potential risk factors were identified in 52 (80%) patients; 23 (36.9%) had multiple risk factors. Trauma remained the main predisposing factor, accounting for 30 eyes (46.2%), followed by preexisting ocular disorders in 18 eyes (27.7%) and systemic disease in 10 eyes (15.4%).

Table 1. Isolated Fungal Genera in Patients with Filamentous Fungal Keratitis

Genus/Species	No. (%)
Fusarium	29 (44.6)
Fusarium solani SC	23 (35.4)
Fusarium oxysporum SC	3 (4.6)
Fusarium dimerum SC	2 (3.1)
Gibberella fujikuroi SC	1 (1.5)
Colletotrichum[*,†]	7 (10.8)
Colletotrichum gloeosporioides SC	5 (7.8)
Colletotrichum truncatum SC	2 (3.1)
Purpureocillium lilacinum	7 (10.8)
Aspergillus	6 (9.2)
Aspergillus versicolor	1 (1.5)
Aspergillus flavus	2 (3.1)
Other	3 (4.6)
Curvularia	4 (6.2)
Curvularia geniculate	3 (4.6)
Curvularia lunata	1 (1.5)
Scedosporum apiospermum	2 (3.1)
Alternaria	1 (1.5)
Bipolaris[*]	1 (1.5)
Corynespora torulosa[*]	1 (1.5)
Lasiodiplodia[*]	1 (1.5)
Lomentospora prolificans	1 (1.5)
Paracremonium contagium[*]	1 (1.5)
Penicillium	1 (1.5)
Phellinus noxius[*]	1 (1.5)
Sarocladium bacillisporum/S. kiliense[‡]	1 (1.5)
Scopulariopsis brevicaulis	1 (1.5)
Total	65 (100)

SC, species complex.

[*]Ten isolates had disagreement of diagnosis between internal transcribed spacer (ITS) and morphological identification, including five *Collectotrichum*.

[†]Two *Collectotrichum* could not be identified by culture.

[‡]ITS could not differentiate these two species.

Treatment and Outcomes

All patients were initially treated with empiric antibiotics and then antifungal agents when their culture findings or clinical manifestations suggested fungal infection. The most commonly used antifungal agents were natamycin, amphotericin B, and voriconazole, which were used as single agents or in combination. As presented in Table 4, 33 (50.8%) patients had resolution of ulcers with medical treatment only, whereas various surgeries were performed in 32 (49.2%) patients. In addition, 6 patients (9.2%) had corneal perforation and 9 patients (13.9%) required

Table 2. Demographics and Clinical Features of Filamentous Fungal Keratitis

Characteristics	No. (%)
Age (years)	
<18	0 (0)
18–65	40 (61.5)
>65	25 (38.5)
Sex	
Male	49 (75.4)
Female	16 (24.6)
Occupation	
Outdoors	29 (44.6)
Indoors	36 (55.4)
Affected Eye	
Right	34 (52.3)
Left	31 (47.7)
Location	
Central	10 (15.4)
Paracentral	40 (61.5)
Peripheral	12 (18.5)
Near total	2 (3.1)
Not documented	1 (1.5)
Size	
Small	17 (25.8)
Medium	34 (51.5)
Large	11 (16.7)
Perforation at presentation	3 (4.5)
Hypopyon	
Yes	24 (36.4)

Table 3. Risk Factors for Filamentous Fungal Keratitis

Risk Factors	No. (%)*
Trauma	30 (46.2)
Pre-existing ocular disorders	18 (27.7)
Systemic disorders	10 (15.4)
Wearing of contact lenses	7 (10.8)
Topical steroid use	7 (10.8)
Recent ocular surgery	1 (1.5)
Unknown	13 (20.0)

*Total is greater than 100% because 23 patients had multiple factors.

therapeutic/destructive surgical interventions such as therapeutic penetrating keratoplasty or evisceration.

BCVA in the affected eye was recorded in 63 eyes at the time of the final follow-up examination (average follow-up time: 19.8 months, ranging from seven days to 113.5 months). Final VA was worse than 20/200 for 21 (32.3%) patients; 26 patients (40.0%) had a VA between 20/200 and 20/40, and 16 patients (24.6%) had a VA equal to or better than 20/40.

Table 4. Treatment for Filamentous Fungal Keratitis

Treatment	No. (%)
Medical treatment	33 (50.8)
Surgical treatment	32 (49.2)
Keratectomy	17 (26.2)
AMT	8 (12.3)
TPK	7 (10.8)
AC irrigation	3 (4.6)
Evisceration	2 (3.1)
Surgical treatment (exclude keratectomy only)*	20 (30.8)
Multiple surgical interventions	5 (7.7)

AC, anterior chamber; AMT, amniotic membrane transplantation; TPK, therapeutic penetrating keratoplasty.

*One of the physicians preferred to do early keratectomy as extensive debridement.

A simple linear regression indicated that six factors were potentially associated with visual outcome converted to logMAR of BCVA: age, presenting time, poor VA at initial presentation, trauma, hospitalization, and antifungal drug usage duration. Other tested variables included sex, size, hypopyon presentation, each predisposing factor, outdoor occupation, treatment duration, and *Fusarium* species did not show any significant association with visual outcome. The multiple linear regression revealed that presenting time, poor VA at initial presentation, and trauma remained significantly associated with visual outcome (Table 5). Among them, trauma was negatively associated with visual outcome.

Discussion

Identification of the fungal isolates using molecular methods provides accurate diagnosis, which may help us better understand the epidemiology and clinical-therapeutic correlation of filamentous fungal keratitis.[12] With molecular identification, we observed changes in trends of isolated filamentous fungal corneal organisms, an emerging incidence of *Colletotrichum* spp. and *P. lilacinum* (10.8%), and diverse pathogens in the current study.

Our study demonstrated that the etiological agents for filamentous fungal keratitis have changed recently. Previous large series having more than 1000 isolates from South India[13–17] and China[18] reported that *Fusarium* spp. (35.0% to 56.9%) and *Aspergillus* spp. (15.3% to 30.7%) were the most and second most

Table 5. Factors Affecting Visual Outcome

	Simple Linear Regression			Multiple Linear Regression		
Variables*	Coefficient†	95.0% CI	*P* Value	Coefficient†	95.0% CI	*P* Value
Age	0.018	0.004–0.032	0.015	0.004	−0.006–0.014	0.394
Presenting time	0.034	0.012–0.056	0.004	0.022	0.008–0.037	0.003
Initial VA in logMAR	0.694	0.465–0.924	0	0.445	0.231–0.659	0
Trauma	−0.885	−1.367 to −0.402	0.001	−0.73	−1.063 to −0.396	0
Hospitalization	0.793	0.287–1.298	0.003	0.244	−0.155 to 0.644	0.226
Antifungal duration (days)	0.018	0.009– 0.027	0	0.007	−0.001 to 0.014	0.08

95% CI, 95% confidence interval.

*Other tested variables included sex, size, hypopyon presentation, each predisposing factor, outdoor occupation, treatment duration, *Fusarium* species. Only significant variables were shown.

†Dependent variable: visual acuity in log MAR at the last follow-up.

common cultured filamentous fungi, which were the same as reports of microbial keratitis in Taiwan.[9] In the current study, *Fusarium* spp. (44.6%) remained the most common cultured pathogens, but *Colletotrichum* spp. and *P. lilacinum* have become second most common.

Colletotrichum spp. are crucial plant pathogens, but their taxonomy remains challenging. Morphological identification of fungal isolates is not only time intensive but also sometimes not straightforward. For example, the falciform conidia of some *Colletotrichum* spp. may mislead inexperienced laboratory personnel and result in them making an incorrect diagnosis of *Fusarium,*[19] which occured through morphological identification in some culture reports for our patients. *Colletotrichum* spp. remaied an infrequent cause, accounting for 1.85% to 3.33% of fungal keratitis diagnosed by smear and cultures,[20–22] and has been reported only once as the fifth most common mold in fungal keratitis (4.1%).[22] We reviewed samples from corneal scrapings that tested positive for molds in our hospital since 2003, and no cases of *Colletotrichum* infection were identified by the end of 2014.[23] Thus we wondered whether accurate diagnosis was made after using molecular identification, which may explain how the incidence of *Colletotrichum* spp. became second most common in our study.

We also observed an increased incidence of *P. lilacinum* (10.8%), which have a reported incidence of approximately 4% to 5% in fungal keratitis.[24] In contrast to the diagnosis of *Colletotrichum* spp., that of *P. lilacinum* was consistent when using either molecular tests or conventional morphological methods. We will continue to observe whether the incidence of *Paecilomyces* keratitis increases.

Our study also observed highly diverse pathogens. In addition to well-established infectious agents such as *Alternaria, Bipolaris, Curvularia,* and *Penicillium*,[25,26] rarely reported species included *Corynespora,*[27] *Lasiodiplodia,*[28] *Scedosporium apiospermum,*[29] *Scedosporium,*[26] *Sarocladium,*[30] and *Scopulariopsis brevicaulis.*[31] Moreover, *Paracremonium* and *Phellinus*, which have never been reported as pathogens of human keratitis, have been found. The results highlighted that using molecular methods may help in the identification of uncommon filamentous fungal agents.[12]

We have to consider the cost-effectiveness and efficiency of diagnostic tools when we determine the level of identification required in a clinical setting. By using morphological characteristics, causative agents can be identified only to the genus level. In some cases, isolates were reported as unidentified dematiaceous and hyaline species, demonstrating that genus-level identification was also difficult. By contrast, the application of polymerase chain reaction (PCR) with sequence determination may provide accurate diagnosis at the species level. Obtaining exact species-specific information may be crucial because the species pattern of the predominant genera can be diverse within a geographical area, and antifungal susceptibility patterns may vary between different species of the same genus.[4]

ITS sequences provide level of identification that appears to hold clinical relevance.[32] ITS sequences are considered to be the universal barcode sequences for fungi because there are more existing fungal ITS sequences than any other genes, a high PCR amplification success rate, and a high degree of interspecific and intraspecific variation.[33] Some have suggested that ITS PCR-based molecular identification should be applied during screening and diagnostic tests when early mycotic keratitis is suspected.[34] It is also noted that ITS segments may be too conserved to distinguish between certain species[4,32]; therefore the use of

other genes, such as elongation factor 1-α, tubulin, GAPDH, actin, and CHS-1 are necessary depending on the species studied.[35] Although ITS region may have some limitations, it could reliably differentiate *Fusarium* spp., the most cultured filamentous fungus, into clinically relevant species complex groupings as indicated in our study. Different susceptibility patterns between species and its implementation in treatment is our next research goal.

Cornea trauma is the most predominant predisposing factor in filamentous fungal keratitis, accounting for 40% to 60% of cases.[7] Our results also showed that nearly half of our patients had trauma. Wearing of contact lenses has become a major risk factor for fungal keratitis, particularly *Fusarium* and *Paecilomyces*/*Purpureocillium* keratitis, in developed countries.[2,36–38] But our results, which indicated that approximately one tenth of the patients wore contact lenses (ranking fourth in risk factors), did not support this trend.

In our study, natamycin and amphotericin B were the most used topical antifungal agents. Voriconazole was third most used, and some patients used oral voriconazole. These results are consistent with the current general practice of using natamycin as the drug of choice against filamentous fungus[39] or amphotericin B as a first line therapy.[4] According to the Mycotic Ulcer Treatment Trial (MUTT) study, natamycin was associated with better clinical outcome than voriconazole for the treatment of *Fusarium* keratitis;[40] cases with suboptimal response to natamycin may benefit from additional oral voriconazole usage.[41] Voriconazole is suggested to be the first-line treatment for *Paecilomyces*/*Purpureocillium* keratitis.[38] There is no consensus regarding therapy for *Colletotrichum* keratitis in the literature.[19,42] With our limited experience, we found that there were different clinical outcomes between *C. gloeosporioides* SC and *C. truncatum* SC; the latter seemed to be more difficult to treat compared to the former.[43] Ten patients with small to medium ulcerations caused by various species infections were not treated with antifungal agents, but the infections resolved, which might be related to the patients' immune responsiveness, a decrease in organism loading after corneal scraping/keratectomy, and the possible antifungal effects of certain antibiotics.[44]

Filamentous fungal keratitis remained difficult to manage, with a high corneal perforation rates and poor visual outcomes noted. Although 32 of the 65 patients in the study required surgical interventions, 17 patients received keratectomy initially because one of the physicians preferred to perform it as early and extensive debridement. After those patients who received only keratectomy were excluded, the surgical rate was 30.8%, which was in between those of previous reports, which ranged from 12.7% to 52.8%.[2,18,23] Apart from poor VA upon presentation, we did not identify any factors, including risk factors and organisms, that predisposed these patients to perforation or impending perforation (data not shown).

In our study, presenting time and poor initial VA were positively, but trauma was negatively associated with poor visual outcome. The MUTT Therapeutic Exploratory Study reported older age, worse presentation visual acuity, larger infiltrate size at presentation, and pigmented ulcer were important prognostic factor for three-month visual acuity.[45] In theory, the location and size of corneal ulcer should affect visual outcome, but we did not get the expected results probably due to relatively small sample size. Trauma being negatively associated with worse visual outcome seems illogical. The association is unclear, although we speculate two possible reasons. First, patients with traumatic events are usually more anxious about their ocular condition and therefore would search for medical attention more eagerly. Second, patients with ocular trauma history, especially with plant matters, would raise the physician's awareness of possible fungal infection and therefore initiate anti-fungal agents earlier. Further studies should be done to confirm our speculation.

The limitations of this study were its retrospective nature, relatively small sample size, and lack of antifungal drug susceptibility test. In addition, physicians had used diverse protocols for treating patients. Moreover, similar to other microbiological studies, our findings should not be generalized to other regions or populations. Although our study design could not demonstrate the advantage of speed in molecular diagnosis, our results revealed that molecular methodology provides insight into accurate identification, which has effects on the epidemiology and diversity of pathogens of filamentous fungal keratitis. In addition, the clinical features of filamentous fungal keratitis provide background information for ophthalmologists practicing in Taiwan.

In conclusion, through molecular identification, we observed a shifting trend of causative filamentous fungus in mycotic keratitis, the increased incidence of the previously uncommon pathogens *Colletotrichum* spp. and *P. lilacinum* as the second-most common causal agents and revealed some previously unknown possible human pathogens. In the future, we intend to deeply understand these etiologic microorganisms, clinical outcomes, and drug susceptibility tests to provide physicians with accurate drug choices according to causative agents, thereby providing successful therapy to patients with filamentous fungal keratitis.

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