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Fungal Spectrum Identified by a New Slide Culture and *In Vitro* Drug Susceptibility Using Etest in Fungal Keratitis

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ABSTRACT *Purpose*: To investigate the spectrum of fungal species causing keratitis and to test antifungal drug susceptibility to each isolate using Etest. *Methods*: Microbial cultures were performed for patients who were clinically diagnosed with fungal keratitis between September 2002 and July 2004. Modified slide culture was established to identify the fungal species of the isolates. Etest (AB BIODISK, Solna, Sweden) was applied to determine the antifungal agent susceptibility of each isolate to itraconazole, fluconazole, and amphotericin B *in vitro*, respectively. *Results*: Among 73 eyes of 73 patients with clinical diagnosis of fungal keratitis, 61 strains of fungi were isolated from 61 eyes. The rate of positive culture was 81.3% of all cases. The spectrum of fungal species involved: 58 (95.1%) isolates of filamentous fungi, including the two most common genera–*Fusarium* (n = 33, 54.1%) and *Aspergillus* (n = 9, 14.8%),–followed by 16 (26.2%) isolates of other genera of filamentous fungi such as *Alternaria* (n = 3, 4.9%), *Trichophyton* (n = 3, 4.9%), *Curvularia* (n = 2, 3.3%), *Chrysosporium* (n = 2, 3.3%), *Acremonium* (n = 2, 3.3%), and *Scedosporium* (n = 1, 1.6%), 1 (1.6%) yeast of *Candida*, as well as two (3.3%) dimorphic fungi of *Blastomyces* and *Sporothrix* isolate each. Three filamentous fungi of the isolates failed to be identified according to the information provided by slide culture. The results of Etest showed that 20 (60.6%) isolates of *Fusarium* were susceptible to amphotericin B, whereas all of them were resistant to itraconazole and fluconazole. All nine (100%) isolates of *Aspergillus* were sensitive to itraconazole, whereas four (44.4%) of them were sensitive to amphotericin B, and only two (22.2%) of them were sensitive to fluconazole. Seventeen (89.5%), 13 (68.4%), and 10 (52.6%) isolates of the remaining 19 organisms were sensitive to amphotericin B, itraconazole, and fluconazole, respectively. *Conclusions*: *Fusarium* and *Aspergillus* are the most frequent pathogenic organisms in causing fungal keratitis, whereas other species of fungi can also cause corneal infection. *In vitro* Etest for assessing antifungal drug susceptibility is a simple and practical method and may provide referential information for clinical consideration of choosing antifungal agents to treat fungal keratitis.

KEYWORDS Etest, fungal identification, fungal keratitis, fungal spectrum, modified slide culture

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INTRODUCTION

The incidence of fungal keratitis has increased in recent years. It has higher morbidity than bacterial or viral keratitis in tropical and subtropical regions. Definite diagnosis and prompt and appropriate clinical managements play important roles in achieving better prognosis in fungal keratitis.[1] Although clinical history and feature of corneal infiltrate are helpful for diagnosing fungal keratitis, definite diagnosis can only be made when pathogen is isolated in the culture of the corneal scraping sample. When pathogen culture is positive, fungal species can be identified according to the macroscopic characteristics of the colony morphology, color and growth rate of the molds, and the microscopic characteristics of hyphae, spores, or conidia, and their relationships. Slide culture is the best method for preserving the actual structure of a fungus. It is not a rapid technique, but it is unsurpassed as a routine means for studying the fine points of microscopic morphology of fungi.[2] In the literature, more than 40 genera, including 70 species of fungi have been reported as pathogenic to human cornea.[3] Filamentous fungi are the principal causes of corneal infections in most parts of the world; either *Fusarium* or *Aspergillus* are the most common fungal genera.[4] However, reports of the spectrum of fungal species in causing keratomycosis are few from China.

Drug susceptibility of the fungal isolates performed *in vitro* may provide useful information for clinical reference in choosing antifungal agents so to improve the prognosis of fungal infection.[5] The National Committee for Clinical Laboratory Standards (NCCLS) Subcommittee on Antifungal Susceptibility Tests has developed standard procedures M27-A2 for yeasts[6] and M38-A for filamentous fungi.[7] These two broth dilution methods are cumbersome and time consuming, and alternative methods have been evaluated in recent years. Among them, the Etest (AB BIODISK, Solna, Sweden) stable gradient MIC (minimal inhibitory concentration) method has been suggested as an alternative approach because of its simplicity and economy. More importantly, good levels of agreement between Etest and NCCLS methods have been well established.[8]

In this study, we developed a new slide culture method for identification of fungi to investigate the spectrum of fungal species in causing keratomycosis in the southeast region of China. Etest is applied to evaluate *in vitro* drug susceptibility of each isolate to itraconazole, fluconazole, and amphotericin B.

MATERIALS AND METHODS

Sample Collection, Inoculation, and Culture

Corneal scraping samples or corneal tissues were collected from patients in the Department of Ophthalmology, Sir Run Run Shaw Hospital a national university hospital located in Hangzhou of Zhejiang province, which provides medical service to all the people in Zhejiang Province residing either in cities or rural areas. Zhejiang province is located in the southeast region of China, in the typical subtropical zone of east longitude of 119 to 121 degrees and north latitude of 28 to 30.5 degrees, and with a population of 43 million.

Initial microbial cultures were performed for 73 cases of patients with clinical diagnosis of fungal keratitis during the period September 2002 to July 2004. The clinical diagnosis was made on the basis of the history of ocular trauma, and corneal infiltrate with gray, elevated necrotic slough, feathery lines extending beyond the ulcer edge in the cornea. The culture materials were collected in two ways. One was from corneal scraping in 70 cases at their first visit in our clinic; the other three samples were obtained from three cases for whom therapeutic penetrating keratoplasty was carried out because of the corneal perforation caused by fungal infection, and the removed recipient corneal tissues were used for pathogen culture. The samples were directly inoculated onto four media, including blood agar, chocolate agar, Sabouraud dextrose agar (SDA), and eosin methylene blue agar. Sabouraud agar plates were incubated at room temperature. The other three plates were maintained at 35°C. The cultures were considered positive for fungus if the same color and morphology of colonies grew on more than two plates.

Modified Slide Culture to Identify the Fungal Species

We developed a new slide culture system for identifying the isolated strains of fungi, which is simpler and more convenient than the procedure of conventional slide culture method developed by Davise.[2] The protocol is described in the following: First, we placed a 20 × 20 mm sterile coverslip onto a SDA plate and

inoculated the fungus of a selected good colony onto the agar adjacent to one angle of the coverslip. The plate was maintained for 2 to 4 days. Second, we used forceps to remove the coverslip carefully when we saw fungi had grown on agar along the margin of the coverslip. We then reversed the coverslip and placed it on a clean slide on which a drop of lactophenol cotton blue (LPCB) was added previously. We covered a larger coverslip (25 × 50 mm) on the slide and sealed the margins with nail polish. Thereafter, we observed and made digital photos under the microscope with low (200×) and high (800×) magnification. Finally, we identified the fungal species according to the morphology, color, and growth rate of the molds, combined with the hyphae, spores, or conidia and their relationships.

Etest Method to Determine *In Vitro* Antifungal Susceptibility

Etest (AB BIODISK, Solna, Sweden) was performed in accordance with the manufacturer's instructions. Etest consists of a thin, inert, and nonporous plastic strip. One side of the strip is calibrated with MIC values in μg/ml. A predefined and exponential gradient of the dried and stabilized antifungal agent is immobilized on the other surface of the strip. After incubation, when growth becomes visible, a symmetrical inhibition ellipse centered along the strip is seen. The zone edge intersects the strip at the MIC value.

Agar Formulation

RPMI 1640 (Gibco BRL, Grand Island, NY, USA) was supplemented with 1.5% agar and 2% glucose and buffered to pH 7.0 with 0.165 M morpholine propane sulfonic acid (MOPS) buffer (Amresco, Solon, OH, USA). Sixty milliliters of RPMI agar were added to 120-mm-diameter plate to reach a depth of 4.0 mm. The plate was then placed at room temperature for 2 days and stored at 4°C for future use.

Preparation of Inoculum

One selected colony was transferred onto a SDA plate incubated for 5–10 days to ensure adequate sporulation. Spore suspensions were prepared in 1 ml sterile saline and adjusted with an ultraviolet-visual light spectrophotometer (Hewlett Packard 8453, Germany) to a concentration of 0.5×10^6 to 5×10^6 CFU/ml, corresponding to the optical densities (OD) ranged from 0.09 to 0.5 at 530 nm.

Etest Procedure

RPMI 1640 agar plates were inoculated by dipping a sterile swab into the spore suspension solution and streaking it across the entire surface of the agar in three directions. The plates were dried for 15 min and Etest strips containing itraconazole (IT), fluconazole (FL), or amphotericin B (AP) were placed onto the inoculated agar, respectively. These three drugs were selected for Etest because they were clinically found to be effective against keratomycosis in a previous study based on clinical observation.[1] The plates were incubated at 35°C, 5% CO_2 for 24–48 hr. The Etest MICs were read directly for the drug concentration at which the border of the elliptical inhibition zone intersected the test strip. According to the NCCLS proposal,[7] the resistance break points for fluconazole, itraconazole, and amphotericin B were defined as $\geq$64 μg/ml, $\geq$1 μg/ml and $\geq$1 μg/ml, respectively.

RESULTS

Spectrum of Fungal Species

Seventy-three eyes of 73 patients were clinically diagnosed with fungal keratitis between September 2002 and July 2004 in our hospital. Sixty-one eyes (81.3%) of 61 cases had positive culture for fungi. Twenty-one species of fungi in 11 genera were involved in this group of patients, including 58 (95.1%) isolates of filamentous fungi, one (1.6%) isolate of yeast, as well as two (3.3%) of dimorphic fungi. The two most common genera were *Fusarium* (n = 33, 54.1%) and *Aspergillus* (n = 9, 14.8%). Figures 1A and 1B show the typical microscopic morphology of *Fusarium solani* and *Aspergillus fumigatus* on the slide culture. Other 16 (26.2%) filamentous species of the isolates included *Alternaria* (n = 3, 4.9%), *Trichophyton* (n = 3, 4.9%), *Curvularia* (n = 2, 3.3%), *Chrysosporium* (n = 2, 3.3%), *Acremonium* (n = 2, 3.3%), *Scedosporium* (n = 1, 1.6%), and three other isolates failed to be identified solely according to the characteristics of slide culture. One isolate of yeast was identified as *Candida albicans*. Two isolates of dimorphic fungi were *Blastomyces* and *Sporothrix*. As far as we know, *Trichophyton verrucosum* (Fig. 1C) and *Sporothrix schenckii* (Fig. 1D) have never been reported as pathogenic organism to cornea. The spectrum of 61 clinical isolates of fungi is summarized in Table 1.

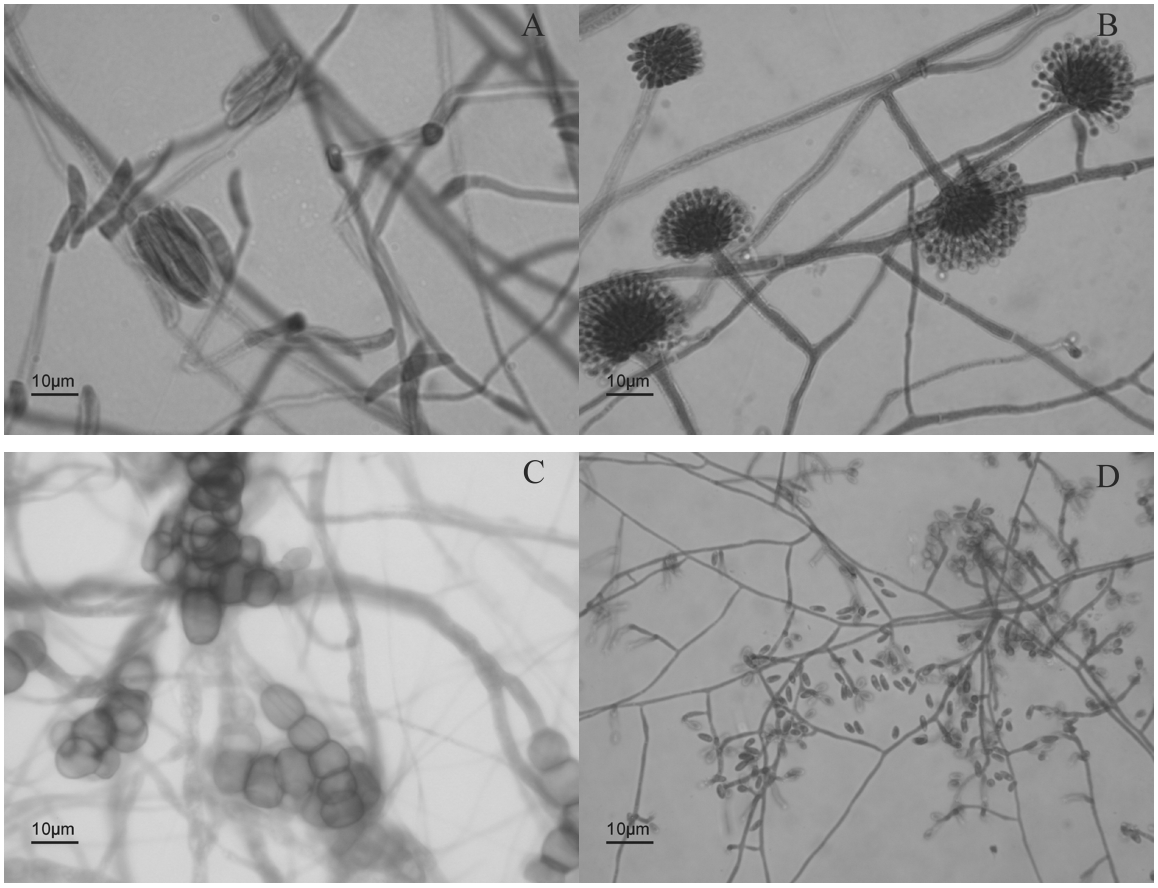

FIGURE 1 **(A) Microscopic characteristics of *Fusarium solani* in slide culture. Septate hyphae are 2–5 μm wide. Macroconidia are arising latterly from hyphae. The cells are moderately curved with blunt apical, mostly three-septate, in size of 2–4 $\times$ 18–42 μm ($\times$800). (B) Microscopic characteristics of *Aspergillus flavus* in slide culture. Septate hyphae are 3.5–5 μm wide. Conidial heads radiate. Conidiophore stipes is rough-walled, hyaline. Spherical vesicles are 25–45 μm in diameter. Conidia spherical or subspherical, 3.5 μm in diameter ($\times$800). (C) Microscopic characteristics of *Trichophyton verrucosum* in slide culture. Chlamydospores are brown color, stringbean-shaped, 8–10 μm in diameter, arranged in chains. Hyphal tips swollen ($\times$800). (D) Microscopic characteristics of *Sporothrix schenckii* in slide culture. Hyphae narrow, 1–2 μm wide, septate, and branching. Conidia are one-celled, small tear-shaped (2.5–5.5 $\times$ 1.5–2.5 μm) on thread-like denticles, forming a rosette-like cluster ($\times$800).**

In Vitro Antifungal Susceptibility to Fluconazole, Itraconazole, and Amphotericin B

Table 1 summarizes the MIC values of drug susceptibilities in Etest of 61 clinical isolates to fluconazole, itraconazole, and amphotericin B, respectively. Among 33 isolates of *Fusarium*, 20 (60.6%) were sensitive to amphotericin B, in which the Etest MICs were lower than 1 μg/ml (MIC range: 0.06 to about 1 μg/ml). However, all isolates of *Fusarium* were resistant to fluconazole and itraconazole (MIC range: 96 to about >256 μg/ml, 8 to about >32 μg/ml, respectively). Figure 2A shows the result of one isolate of *Fusarium solani* that has been examined for its antifungal agent susceptibility in Etest. For this isolate of *Fusarium solani*, the Etest MICs were 0.5 μg/ml to amphotericin B, more than 256 μg/ml to fluconazole, and more than 32 μg/ml to itraconazole, respectively.

The MICs of fluconazole, itraconazole and amphotericin B against *Aspergillus* were varied from 8 μg/ml to >256 μg/ml, 0.01 μg/ml to 0.50 μg/ml, and 0.02 μg/ml to >32 μg/ml, respectively. All nine (100%) isolates of *Aspergillus* were sensitive to itraconazole, whereas four of them (44.4%) were sensitive to amphotericin B, and only two of them (22.2%) were sensitive to fluconazole. Figure 2B illustrates one specimen of *Aspergillus flavus* that was sensitive to itraconazole and amphotericin B but resistant to fluconazole.

The MICs of these three drugs against the remaining 19 organisms varied depending on the species. Among these isolates, 17 (89.5%) isolates were sensitive

TABLE 1 Spectrum of Fungi in Causing Keratomycosis and Drug Susceptibility to Fluconazole, Itraconazole, and Amphotericin B in Etest

Genus	Species	Number of isolates	MICs (μg/ml) in Etest		
			FL	IT	AP
Fusarium	*Fusarium solani*	27	96 ~ ≥256	8 ~ ≥32	0.06 ~ ≥32
	Fusarium oxysporum	5	≥256	≥32	0.13 ~ ≥32
	Fusarium spp.	1	≥256	≥32	≥32
Aspergillus	*Aspergillus flavus*	5	32 ~ ≥256	0.09 ~ 0.50	0.09, ≥32
	Aspergillus fumigatus	2	8, ≥256	0.02, 0.03	0.02, 0.13
	Aspergillus niveus	1	≥256	0.01	1.0
	Aspergillus terreus	1	≥256	0.50	≥32
Trichophyton	*Trichophyton mentagrophytes*	2	192, ≥256	0.25, 0.75	0.01, 0.10
	Trichophyton verrucosum[a]	1	1.50	0.19	0.16
Alternaria	*Alternaria longipes*	2	32, ≥256	0.25, 0.75	0.01, 0.10
	Alternaria alternata	1	12	0.75	0.01
Curvularia	*Curvularia geniculata*	1	6	0.01	0.01
	Curvularia brachyspora	1	8	0.05	0.16
Acremonium	*Acremonium strictum*	1	≥256	≥32	0.38
	Acremonium kiliense	1	≥256	≥32	0.50
Chrysosporium	*Chrysosporium inops*	1	12	0.16	0.02
	Chrysosporium spp.	1	32	0.19	0.02
Scedosporium	*Scedosporium prolificans*	1	≥256	≥32	≥32
Blastomyces	*Blastomyces dermatitidis*	1	192	4	0.25
Sporothrix	*Sporothrix schenckii*[a]	1	0.50	0.13	0.50
Candida	*Candida albicans*	1	0.25	0.09	0.19
Unknown[a]		3	0.02 ~ ≥256	0.13 ~ ≥32	0.01 ~ ≥32
Isolates in total		61			

FL, fluconazole; IT, itraconazole; AP, amphotericin B; MIC, minimal inhibitory concentration.
[a]Species of fungi have not been previously reported in causing keratomycosis.

to amphotericin B, 13 (68.4%) isolates were sensitive to itraconazole, and 10 (52.6%) isolates were sensitive to fluconazole.

The number and percentage of *Fusarium*, *Aspergillus*, and other fungal isolates that were susceptible or resistant to itraconazole, fluconazole, or amphotericin B are summarized in Figure 3.

DISCUSSION

To date, identification of a fungal species still mainly depends on its macroscopic and microscopic morphology in the laboratory culture. The exact judgment closely relies on accurate records of the undisturbed morphology, color, and rate of growth of the molds in combination with characteristics of hyphae, spores, or conidia and their relationships of the isolate growing on the media. Slide culture is the best method to preserve and observe the natural structure of a fungus. Classical procedure used previously was to inoculate the fungus onto the center of agar block on a sterile slide and covered by a coverslip, and then incubate in a plate that is filled with saline. Although growth of the fungus can be examined periodically by this method, it is hard to remove the fungus for staining. We modified the protocol to establish a new slide culture in the current study. We inoculated the fungus onto the Sabouraud agar adjacent to the angle of a sterile coverslip. The fungus then migrates along the margin and grows on the inner surface of the coverslip. After carefully removing the coverslip and staining with LPCB, we could observe the clear and sharp picture of hyphae and spores with undisturbed structure and relationships under microscope (Fig. 1). It is needed to mention that our modified slide culture method is valuable and easy for handling to identify molds and dimorphic fungi but not for yeasts.

Identification of the fungal organisms isolated from clinical cases is important, not only for studying the spectrum of fungi in causing keratomycosis but also for collecting evidence to select susceptible antifungal agents for medical treatment. Geographical and

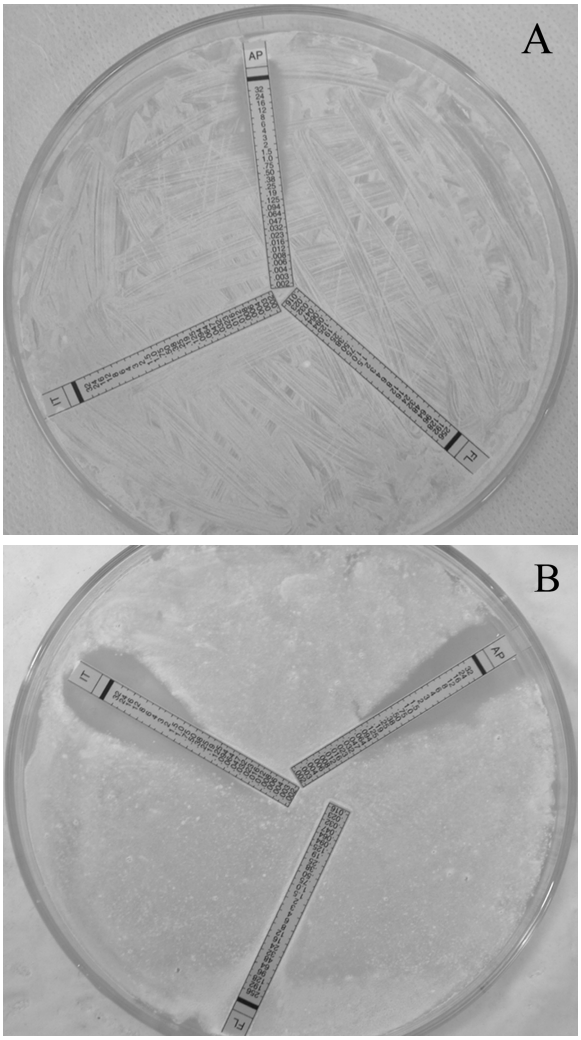

FIGURE 2 **(A) Result of an isolate of *Fusarium solani* in Etest. The MICs were read according to the extent of inoculum growth around the strips that contained antifungal agents of amphotericin B (AP), fluconazole (FL), and itraconazole (IT), respectively. It was clearly demonstrated that this isolate is sensitive to amphotericin B (seen at the peripheral one-third area around the AP strip without growth of inoculum) but resistant to fluconazole and intraconazole (the surrounding areas of FL strip and IT strip were fully occupied by the growth of the inoculum). (B) Result of an isolate of *Aspergillus flavus* in Etest. The MICs were read according to the extent of inoculum growth around the strips that contained antifungal agents of amphotericin B (AP), fluconazole (FL), and itraconazole (IT), respectively. It was clearly demonstrated that this isolate is sensitive to itraconazole and amphotericin B (seen around IT strip and AP strip without growth of inoculum) but resistant to fluconazole (the surrounding area of FL strip was fully occupied by the growth of the inoculum).**

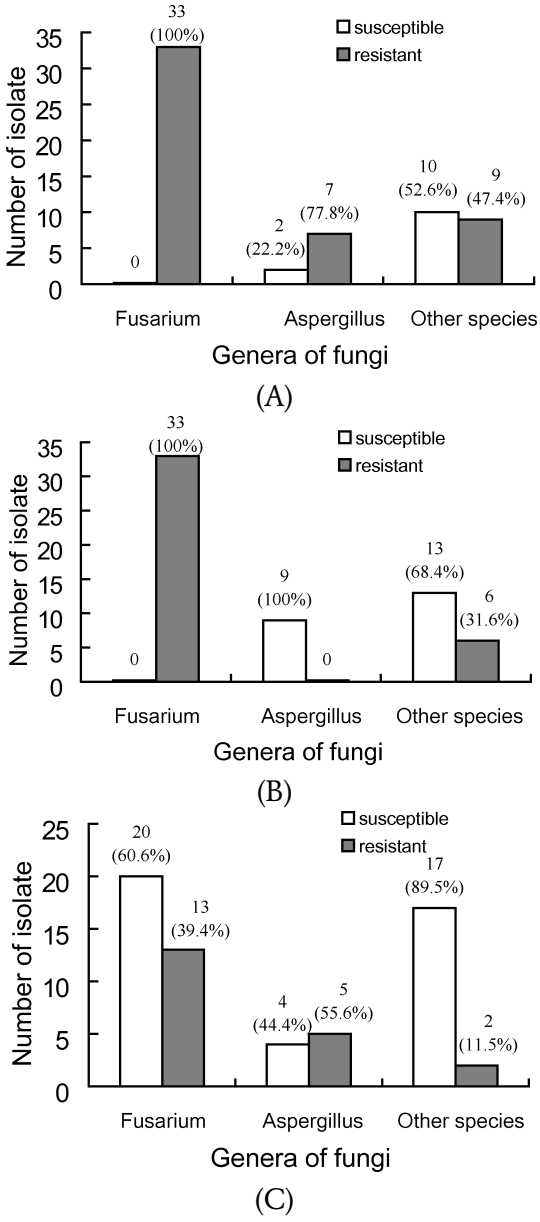

FIGURE 3 **The results of fungal genera isolated from this case series and drug susceptibility of each isolate in Etest were described in the text in detail. It was demonstrated in Etest that 100% of 33 *Fusarium* isolates was resistant to both fluconazole (A) and itraconazole (B), whereas 60.6% was sensitive to amphotericin B (C). Regarding *Aspergillus* of 9 isolates, 22.2% was sensitive to fluconazole (A) and 100% was sensitive to itraconazole (B) in Etest.**

environmental variations, such as humidity and air temperature, have been observed in the predominant genera of fungi isolated from patients with fungal keratitis. In our case series, *Fusarium* was the most frequently isolated pathogen in 33 eyes (54.1%), and *Aspergillus* was the second most often one in 9 cases (14.9%). There are some agreements of this study on the spectrum of fungal species in causing fungal keratitis with others reported from India and the southern United States.[9,10] We also isolated nine other infrequent genera including *Curvularia*, *Alternaria*, *Trichophyton*, *Chrysosporium*, *Acremonium*, *Scedosporium*, *Blastomyces*, and *Sporothrix*, as well as one *Candida* from these patients. To our best knowledge, we are the first to report *Trichophyton verrucosum* and *Sporothrix schenckii* as the pathogenic organism in causing keratomycosis. Additionally, there were three

other isolates from this case series that could not be identified by slide culture method, which have neither been described in literature nor reported previously. We have stocked these three unknown isolates for further study.

With regard to antifungal drug therapy for fungal infectious disease, antifungal drug susceptibility testing has become especially important as a result of the increasing infectious cases and the problem of drug resistance. Some investigators have demonstrated that Etest is a reproducible method for testing antifungal drug susceptibility to molds.[11,12] The results from Etest and the standardized broth microdilution method (M38-P) have shown to be more than 80% in agreement for molds and 100% agreement for *Fusarium* and *Aspergillus*.[11,12] Besides, Etest has its own advantages of reliable, accurate, and intuitionistic reading of the results, and is economical and easy for handling. Inoue et al.[13] introduced the Etest for isolates from three cases of fungal keratitis. We report herein for 61 isolates from 61 cases using the same testing procedure.

The result showed that *Fusarium* was susceptible to amphotericin B in 20 (60.6%) of 33 isolates but resistant to both fluconazole and itraconazole in 100% of the 33 isolates. This result suggests amphotericin B may be helpful for treatment of *Fusarium* keratitis. Although systemic use of amphotericin B is restricted due to its significant nephrotoxicity and poor penetrability, amphotericin B eyedrops in topical use or combined with other antifungal agents should be considered as medical choice.

There were 15 isolates (*Fusarium*, 13; *Scedosporium prolificans*, 1; unknown fungus, 1) demonstrated to be resistant *in vitro* to all three drugs: itraconazole, fluconazole, and amphotericn B. Among them, 2 eyes of 15 cases (one infected by *Fusarium* and the other by unknown fungus) were enucleated due to failure in controlling the corneal infection or severe ocular complications (data not shown). Two *Fusarium* keratitis patients refused medical treatment because of economic problems. Finally, among the other 10 *Fusarium* keratitis patients, one was successfully treated by oral itraconazole only, and 9 of them needed therapeutic penetrating keratoplasty with finally preserved eyeball integrity and eradication of the infection after surgery (data not shown). The result of *Fusarium* isolates resistant to triazoles *in vitro* in this study is agreement with that reported by others.[14,15] These results of drug susceptibility test and clinical outcomes suggest that it is necessary to look for other antifungal agents for medical treatment of these drug-resistant keratomycosis.

In this study, we found that all 9 *Aspergillus* isolates (100%) were sensitive to itraconazole, whereas only four (44.4%) of them were sensitive to amphotericin B, and only two (22.2%) of them were sensitive to fluconazole. This is in agreement with previous reports.[14,16,17] Clinically, eight *Aspergillus* keratitis patients were successfully treated by oral itraconazole only, and one needed therapeutic penetrating keratoplasty (data not shown). The results of drug susceptibility test and clinical outcomes suggest that itraconazole can be considered as the first choice of medical treatment of *Aspergillus* keratitis.

One point that should be mentioned here is that fungal isolate susceptibility or resistance to antifungal agents is based on the drug concentration in the blood. However, the fact is that there is a discrepancy of concentration between the blood and the corneal tissue or aqueous humor. Additionally, the clinical response is also associated with factors such as timing of antifungal agent initiated, type of drugs applied, the host immune response, and the virulence of the pathogen.[18] We are currently carrying out an *in vivo* study using rabbit keratomycosis model to investigate the exact correlation between Etest-MIC and clinical outcome after antifungal agent treatment. Meanwhile, the clinical courses and final results of these 61 patients after clinical managements including antifungal agent treatment will be further analyzed and reported elsewhere. It is anticipated that a definite significance of Etest results for the referential choosing of antifungal agents in the treatment of keratomycosis will be clarified in the future.

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