·Letter to the Editor·

The pathogenic spectrum of fungal keratitis in northwestern China

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Dear Editor,

I am Na An, from the Shaanxi Key Lab of Ophthalmology, Shaanxi Institute of Ophthalmology, Xi'an City First Hospital, Xi'an, Shaanxi Province, China. Fungal keratitis is a severe problem in most developing countries. It is one of the most serious corneal infectious diseases and can lead to blindness [1]. Studies conducted in various regions of China and India have shown that the most common fungal pathogen is *Fusarium* spp., followed by *Aspergillus* spp. In rare cases, *Curvularia*, *Alternaria*, *Penicillium* or *Candida* can be the causative pathogen. Variation in the endemic species of causative pathogen, including specific portions and rare filamentous fungi, has been observed by region [2-10]. Each year, a large number of patients with severe keratitis are treated at our hospital in northwestern China. We have collected data on the pathogenic fungi present in cases of corneal disease for five years and have used polymerase chain reaction (PCR) and sequencing to identify the genus and species of the pathogens.

From October 2010 to September 2015, 1090 cases of patients definitively diagnosed of fungal keratitis were collected. Of the 1090 patients, 731 (67.1%) were men and 359 (32.9%) were women, the male-to-female ratio was nearly 2:1. Males were dramatically more than females may be attributed to that more men were engaged in the jobs which easier to injure their eyes. Patients between 60 and 69 years old (29.3%) were most, followed by the 50-59 year-old subgroup (27.8%) (57.1% for both groups). The age and gender distribution is showed in Figure 1. The data indicated that in northwestern China, fungal keratitis was occurred more often between the months of October to May, eliminated significantly in summer time. This trend is clearly performed in Figure 2.

A total of 1090 corneal scrapings from the leading edge to the base of the ulcer of each patient were smeared directly onto glass slides for potassium hydroxide (KOH) wet mounting, the discovery of fungal hyphae or spores under a microscope was considered a definitive diagnosis of fungal keratitis, and another copy of the scraping was cultured in Sabouraud's liquid medium and incubated at 28℃. After 48h, the culture tubes were examined every day for growth. Of the 1090 samples, 550 were culture-positive and 540 were culture-negative (positive rate: 50.5%). The hyphae of the positive cultures were ground using an electric mini grinder in a tube on ice for 10s after freezing with liquid nitrogen. The powder was then applied for the extraction of genomic DNA using a DNeasy plant mini kit (Qiagen, Germany) according to the manufacturer's instructions.

The DNA was used to amplify both ITSI and ITSII along with the 5.8S rRNA gene by using primers ITS1 and ITS4 [11]. A 25 μL reaction mixture contained 12.5 μL of 2 × MasterMix PLUS [including deoxynucleotide triphosphates (dNTPs), PCR buffer, Mg^{2+} and Taq polymerase] (Tiangen, China), 1 μL of 10 μmol/L of each of the primers, 2 μL of template DNA sample, and 8.5 μL of ddH_2O. The reaction involved initial denaturation at 95℃ for 10min, followed by 30 cycles in a series of denaturation at 95℃ for 30s, annealing at 50℃ for 30s, and extension at 72℃ for 30s, and a final step of 1 cycle at 72℃ for 10min to final extension. The positive control was the DNA extracted from the standard strain of *Aspergillus fumigatus* purchased from Shaanxi Institute of Microbiology. The negative control was ddH_2O in place of the template DNA. The amplified products (approximately 500 bp, Figure 3) were sufficient for sequencing if the template DNA samples were extracted from the positive cultures of fungal keratitis. The amplified products from the digested corneal tissues (negative cultures) were not enough for sequencing, necessitating a second set

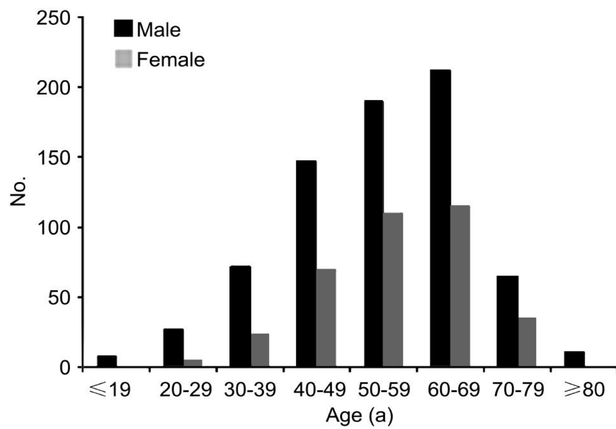

Figure 1 Age and gender distribution of fungal keratitis patients.

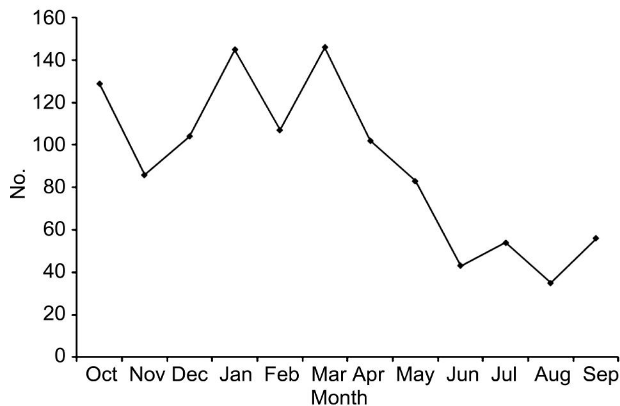

Figure 2 Monthly distribution of the 1090 fungal keratitis patients.

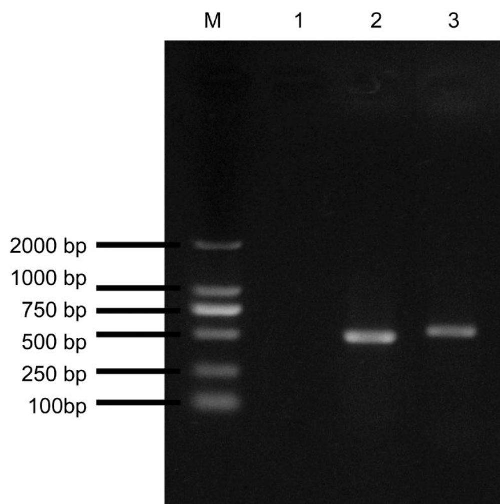

Figure 3 The product of the first set of semi-nested PCR amplified ITS region of the pathogenic fungus M: Marker DL2 000 (TaKaRa, Japan); 1: Negative control (ddH_2O); 2: Positive control (*Aspergillus fumigatus*); 3: Clinical fungal keratitis specimen.

of PCR with the primers of ITS86 and ITS4 [11] (approximately 250 bp, Figure 4), even some samples were still insufficient for sequencing. The component and procedure of PCR was the same as the first set except for the primer (ITS86 instead of ITS1) and the template (the product of the first set of PCR).

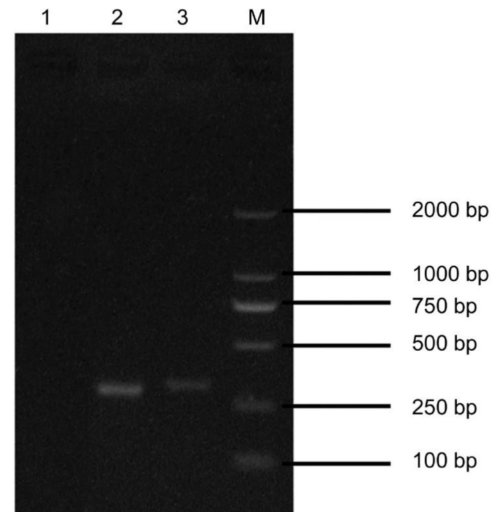

Figure 4 The product of the second set of semi-nested PCR amplified ITS region of the pathogenic fungus M: Marker DL2 000 (TaKaRa, Japan); 1: Negative control (ddH_2O); 2: Positive control (*Aspergillus fumigatus*); 3: Clinical fungal keratitis specimen.

The amplified products obtained with primer pair ITS1 (or ITS86) and ITS4 [11] were sequenced with an ABI Prism automated DNA sequencer (model 3100, version 3.0; Applied Biosystems, Warrington, United Kingdom) with the single primer ITS1 or ITS86. These sequences were used to identify the fungi with the help of the BLAST program (www.ncbi.nlm.nih.gov/BLAST).

The number and percentage of each pathogen identified from the positive cultures is reported in Table 1. Of the 540 negative cultures, 53 were *Fusarium solani*, 5 were *Fusarium verticillioide*, 2 were *Alternaria alternate*, and 1 was *Neurospora crassa*, others were failed to extract enough DNA for PCR and sequencing.

We found that the main causative agent of fungal keratitis in northwestern China was similar to those reported before from other parts of the world and with some differences simultaneously[2-10]. In northwestern China, the most frequent pathogens resulting in fungal corneal infection were *Fusarium*(47.1%), followed by *Aspergillus*(34.7%), *Candida* (6.5%) and *Penicillium* (3.8%), in addition rare cases caused by other species (7.9%) such as *Alternaria*, *Cladosporium*, *Scedosporium*. *Curvularia* and *Chrysosporium* [5-6] were not found. Future studies of diagnosis and treatment methods should therefore focus on *Fusarium* and *Aspergillus*.

The culture method remains the gold standard for diagnosing fungal infection worldwide, with a positive rate of approximately 50%[12-14]. Smear examination is still the most efficient and intuitive way to detect fungal infection, and has the benefit of a higher positive rate than the culture method [12-14]. In our experience, specimens found in corneal scrapings that exhibit many fungal hyphae under a microscope have a high positive rate of PCR detection, but

Table 1 Distribution of pathogens in fungal keratitis

Pathogen	No.	Percent (%)
Fusarium genus	279	50.7
F. solani	198	36
F. oxysporum	43	7.8
F. verticillioides	17	3.1
F. subglutinans	13	2.4
F. proliferatum	4	0.7
F. sporotrichioides	3	0.5
F. delphinoides	1	0.2
Aspergillus genus	188	34.2
A. flavus	123	22.4
A. fumigatus	39	7.1
A. sydowii	17	3.1
A. nidulans	5	0.9
A. niger	4	0.7
Candida genus	36	6.6
C. albicans,	27	4.9
C. parapsilosi	7	1.3
C. glabrata	2	0.4
Penicillium genus	21	3.8
Alternaria genus	16	2.9
A. alternata	11	2
A. tenuissima	5	0.9
Others	10	1.8
Neocosmospora rubicola	2	0.4
Cladosporium cladosporioides	1	0.2
Cladosporium herbarum	1	0.2
Scedosporium apiospermum	1	0.2
Scedosporium prolificans	1	0.2
Trichoderma longibrachiatum	1	0.2
Acremonium strictum	1	0.2
Sporotrichum thermophile	1	0.2
Lichtheimia ramose	1	0.2

specimens that presented few hyphae or spores are difficult to detect by PCR. We therefore conclude that the PCR method is a useful supplement for the diagnosis of pathogens, especially for the identification of previously cultivated pathogens. Furthermore, PCR allows the detection and identification of rare species such as *Scedosporium apiospermum*, *Trichoderma longibrachiatum*, and *Acremonium strictum*, in addition, atypical pathogens exhibiting phenotypic variation have been identified efficiently by PCR. The sensitivity of the PCR method is very high, allowing detection from a small quantity of fungal pathogen DNA. However, determining the genus and species requires a concentrated PCR product; the fungal hyphae and spores found deep in the corneal stroma have hard cell walls that are difficult to break, and the quantity of genomic DNA that is released is typically not sufficient for PCR and sequencing.

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REFERENCES

1 Thomas PA, Kaliamurthy J. Mycotic keratitis:epidemiology, diagnosis and management. *Clin Microbiol Infect* 2013;19(3):210-220.

2 Kim E, Chidambaram JD, Srinivasan M, Lalitha P, Wee D, Lietman TM, Whitcher JP, Van Gelder RN. Prospective comparison of microbial culture and polymerase chain reaction in the diagnosis of corneal ulcer. *Am J Ophthalmol* 2008;146(5):714-723.

3 Bandyopadhyay S, Das D, Mondal KK, Ghanta AK, Purkrit SK, Bhasrar R. Epidemiology and laboratory diagnosis of fungal corneal ulcer in the Sundarban Region of West Bengal, eastern India. *Nepal J Ophthalmol* 2012;4(1):29-36.

4 Nath R, Baruah S, Saikia L, Devi B, Borthakur AK, Mahanta J. Mycotic corneal ulcers in upper assam. *Indian J Ophthalmol* 2011;59(5):367-371.

5 Kredics L, Narendran V, Shobana CS, Vágvölgyi C, Manikandan P; Indo-Hungarian Fungal Keratitis Working Group. Filamentous fungal infection of the cornea: a global overview of epidemiology and drug sensitivity. *Mycoses* 2015;58(4):243-260.

6 Qiu WY, Yao YF, Zhu YF, Zhang YM, Zhou P, Jin YQ, Zhang B. Fungal spectrum identified by a new slide culture and in vitro drug susceptibility using etest in fungal keratitis. *Curr Eye Res* 2005;30(12):1113-1120.

7 Xie L, Zhai H, Zhao J, Sun S, Shi W, Dong X. Antifungal susceptibility for common pathogens of fungal keratitis in Shandong Province, China. *Am J Ophthalmol* 2008;146(2):260-265.

8 Xuguang S, Zhixin W, Zhiqun W, Shiyun L, Ran L. Ocular fungal isolates and antifungal susceptibility in northern China. *Am J Ophthalmol* 2007; 143(1):131-133.

9 Gajjar DU, Pal AK, Ghodadra BK, Vasavada AR. Microscopic evaluation, molecular identification, antifungal susceptibility, and clinical outcome in fusarium, Aspergillus and, dematiaceous keratitis. *Biomed Res Int* 2013; 2013:605308.

10 Wang L, Sun S, Jing Y, Han L, Zhang H, Yue J. Spectrum of fungal keratitis in central China. *Clin Exp Ophthalmol* 2009;37(8):763-771.

11 Ferrer C, Colom F, Frasés S, Mulet E, Abad JL, Alió JL. Detection and identification of fungal pathogens by PCR and by ITS2 and 5.8S ribosomal DNA typing in ocular infections. *J Clin Microbiol* 2001;39 (8):2873-879.

12 Gaudio PA, Gopinathan U, Sangwan V, Hughes TE. Polymerase chain reaction based detection of fungi in infected corneas. *Br J Ophthalmol* 2002;86(7):755-760.

13 Vengayil S, Panda A, Satpathy G, Nayak N, Ghose S, Patanaik D, Khokhar S. Polymerase chain reaction-guided diagnosis of fungal keratitis: a prospective evaluation of its efficacy and limitations. *Invest Ophthalmol Vis Sci* 2009;50(1):152-156.

14 Dalmon C, Porco TC, Lietman TM, Prajna NV, Prajna L, Das MR, Kumar JA, Mascarenhas J, Margolis TP, Whitcher JP, Jeng BH, Keenan JD, Chan MF, McLeod SD, Acharya NR. The Clinical differentiation of bacterial and fungal keratitis: a photographic survey. *Invest Ophthalmol Vis Sci* 2012;53(4):1787-1791.