

Epidemiology of Fungal Keratitis in North Vietnam

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Abstract

The epidemiological characteristics and the causative agents of fungal keratitis at the National Ophthalmology hospital, the biggest eye care hospital in North Vietnam were described by using questionnaire and analyzing of ITS region sequences. In 2008, 687 fungal keratitis patients were diagnosed and 363 fungal strains were isolated from these patients. The predominant fungal species isolated was *Fusarium* spp. (39.6%), followed by *Aspergillus* spp. (25.9%). No yeast species was isolated. A large proportion of the patients was in the middle decades of life (41-60 years; 51.8%) and worked as farmers in rice fields. Corneal ulceration most often occurred after a superficial corneal injury with organic materials. Medical treatment, especially antibiotic use, were started on 551 (80.1%) of the 687 patients. The study findings have important public health implications for treatment and prevention of fungal keratitis in Vietnam.

Keywords: Fungal keratitis; ITS; Vietnam

Abbreviations: ITS: Internal Transcribed Spacer; DDBJ: DNA Data Bank of Japan

Introduction

Corneal ulceration, especially infectious corneal diseases, is one of the leading causes of visual loss and blindness in developing countries, second only to cataract [1,2].

Among infectious agents causing keratitis, fungal agent is responsible for a significant burden of blinding disease in the developing countries. Indeed, the incidence of fungal keratitis has increased dramatically over the past 30 years, with some authors reporting up to 17-44% of keratitis cases caused by fungi [3-5].

Considering the importance of fungal keratitis, studies on etiological factors predisposing to fungal keratitis in Vietnam published in English up to date are surprisingly negligible. Very few studies on this matter in Vietnamese can be found but the information on causative agent characteristics is limited. Obviously, the epidemiological pattern of fungal keratitis differs widely from region to region related to socioeconomic and climate factors.

The National Ophthalmology hospital in Vietnam is the biggest referral center and graduate and post graduate training institute that provides eye care for patients all over the North Vietnam. Every month, approximately 50 mycotic keratitis patients were hospitalized. The identification of fungal agents in clinical laboratories is only based on morphologic characteristics. Conventional mycological identification can be elusive in some cases, especially when morphological characteristics are not easily differentiated. Rapid and precise identification of the infectious agent is of epidemiological importance in medical mycology, as the emergence of unconventional fungal pathogens constitutively resistant to some antimycotic drugs and the broadening of the antimycotic panel have made it an important step in the choice of an effective therapy [3]. Early detection of causative agents for initiation of treatment is pivotal as infections on the most superficial layer well respond to antimicrobials. If the treatment is tardy, infection will advance the inner-most layer, which makes the compromise or loss of vision inevitable [6]. Up to date, in Vietnam, the lack of understanding on epidemiological features and fungal

pathogens causing keratitis obviously restricts ability of effective diagnosis, prophylaxis and treatment of causative fungi.

The aim of this study was to identify the causative agents and describe epidemiological characteristics of fungal keratitis presenting at the National Ophthalmology hospital in the North Vietnam.

Materials and Methods

Patients

All patients with clinically diagnosed fungal keratitis and microbiologically confirmed by fungal hyphae observed in corneal specimen stained on microscopic examination or fungal growth at site of inoculation on solid culture media at the National Ophthalmology hospital from 1st January to 31st December 2008. Studied patients were evaluated for demographic data, duration of symptoms, predisposing factors associated ocular conditions, history of drug use. Only patients with signed consents were enrolled in this study. The protocol of this study was submitted for review to the ethical committees of: (i) National Ophthalmology Hospital and (ii) The Vietnam National Foundation for Science and Technology Development (NAFOSTED).

Fungal identification by sequencing analysis

After growth, lysates for DNA extraction were prepared from approximately 1 cm² mycelia. Fungal DNA was extracted by using MasterPure™ DNA Purification kit (Epicentre, USA) following the manufacturer's instruction. Human DNA from whole blood, DNA from *P. aeruginosa*, *E.coli* and *S. pneumoniae* were used as negative controls and DNA of *Candida albicans* ATCC 26790 was positive

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control. The universal primers of ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'), targeting the conserved regions of 18S, 5.8S, and 28S rDNA were used for amplification [7] (Alpha DNA, Canada). PCR reaction was performed according to Ferrer et al. with some modifications [7]. Amplification reactions contained 1×PCR buffer, 0.2 mM of each dNTP, 0.1 U Taq polymerase (Takara Shuzo Co., Ltd., Otsu, Shiga, Japan), 0.4 μM of each primer, and 5 μl of the template in a final reaction volume of 25 μl. PCR amplification was carried out in a thermal cycler (GeneAmp® PCR system 9700; PE Applied Biosystems, Tokyo, Japan) as follows: 5 min initial denaturation step, followed by 35 cycles of 94°C for 30 s, 54°C for 1 min, and 74°C for 1 min, with a final extension step of 6 min at 72°C. Every reaction positive and negative controls were carried out simultaneously with fungal isolates. Amplified products were examined by agarose gel electrophoresis (1.2%) and ethidium bromide staining. Purified PCR products were sequenced with the use of a BigDye™ Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems) in a PRISM 3100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions.

The sequences of fungal ITS (Internal transcribed spacer) regions were subjected to BLAST analysis to identify at the species and/or genera level. Fungal isolate that its sequence yielded a percent identity of ≥ 99% to a type/validated strain was identified to species level/species complex level. The genus level was assigned if the sequence identity was 95-98% [8,9].

Results

Epidemiologic features

During the study period of one year, within 1153 keratitis patients admitted to the hospital, 687 patients were diagnosed fungal keratitis. Of the total 687 patients 384 (55.9%) were female and 303 (44.1%) were male with fungal keratitis occurring in both groups most frequently in the middle decades of life (Figure 1). The predominance of fungal keratitis in females was most distinct in the middle decades with an overall ratio of female to male patients of 1.3 to 1. Concerning seasonal variation in the incidence, the peak occurred in the months of October to December (Figure 2).

The occupation of the patients reflexes relatively exactly the occupational structure of Vietnam which is an agricultural country (Table 1). The majority were farmers, usually working in rice fields.

The duration from onset of illness to coming to the National Ophthalmology hospital of all patients was given in (Table 2). Most of the patients (262 patients, 38.1%) were examined within 7 days after onset of their illness. However, there were 84 patients coming to hospital after 1 to 2 months and 5 patients waited for more than 2 months.

Medical help from public hospitals and private clinics was sought by 567 (82.5%) of 687 patients before their initial examination at the National Ophthalmology hospital. One hundred twenty remaining patients (17.5%) either sought medical help from pharmacists/village healer (83; 12.1%) or did not get any medical help (37; 5.4%).

Medical treatment was documented in 640 (93.2%) of the 687 patients before coming to the National Ophthalmology hospital. A significant number of patients (120; 17.5%) purchased medication without physician examination and prescription. Of the 640 patients who used prior medication, 523 (76.1%) used either topical antibiotics alone or combination of topical and systemic antibiotics, mostly

ofloxacin and tobramycin; 18 (2.6%) used topical antibiotics combined with corticosteroid, usually polydexa, tobradex; 10 (1.4%) took topical antifungal drugs, natamycin, fluconazole, amphotericin B; and 89 (13%) dropped medication but did not know what kind of drug they used. Thirty seven (5.5%) patients did not use any kind of medication. The traditional remedies were used to treat 10 (1.4%) patients. The popular traditional remedies were eye sauna by betel, bamboo leaves or rose petals and eye washing by high concentration of sodium chloride solution. In some cases, the village healer applied powder of roasted given plant root to the eyes. It is remarkable that five patients with corneal foreign bodies before ulceration tried to remove the foreign body using bamboo bud by themselves or their relatives.

Associated conditions

Histories of recent corneal trauma of 576 (83.8%) patients were obtained and analyzed. Agricultural and vegetable products (254; 44.1%) were major agents responsible for corneal trauma, followed by dust, soil and stone agents (142; 24.7%) (Table 3). Other complicating conditions being able to predispose corneal trauma were found in 35 (5.1%). Those conditions included post corneal surgery (6 patients), prior herpes simplex keratitis (12 patients) and dry eye (17 patients).

Pathogenic agent identification

Positive cultures that fulfilled the criteria for fungal keratitis were 363 (52.8%) of the 687 scraping specimens from 687 patients diagnosed fungal keratitis. Among these isolated fungal strains, ITS regions of 351 isolates were successfully amplified (Figure 3) and sequenced. After trimming primer regions, a consecutive stretch of ITS regions were subjected to compare with BLAST database for identification of fungal isolates. PCR-base sequencing identified isolates belonging to

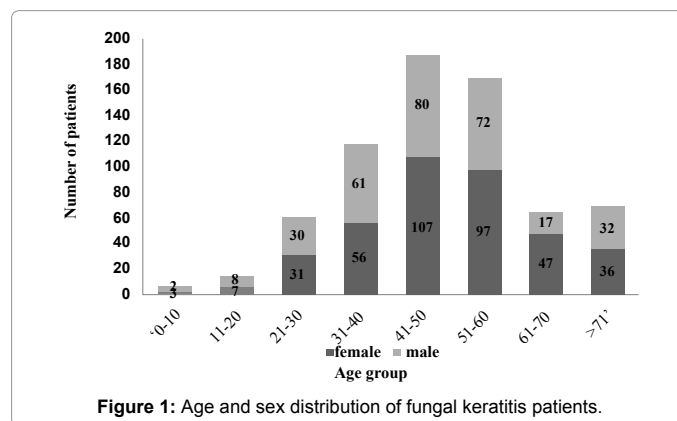


Figure 1: Age and sex distribution of fungal keratitis patients.

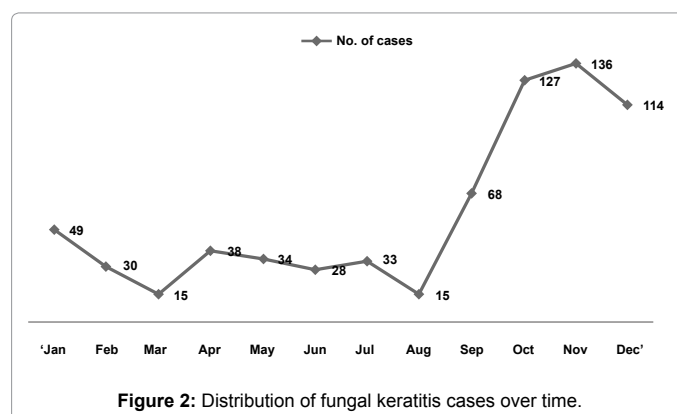


Figure 2: Distribution of fungal keratitis cases over time.

Occupations	No of cases	Percentage
Agricultural worker	486	70.7
Household	80	11.6
Manual labor	51	7.4
Nonmanual labor	26	3.8
Pupil/Student	24	3.5
Unemployed	13	1.9
Deskjob	7	1.0
Total	687	100.0

Table 1: Occupations of fungal keratitis patients.

Duration of symptoms	No of cases	Percentage
1- 7 days	262	38.1
8- 14 days	178	26.0
15- 29 days	158	23.0
1-2 months	84	12.2
>2 months	5	0.7
Total	687	100.0

Table 2: Duration of symptoms in fungal keratitis patients.

Traumatic agent	No of cases	Percentage
Paddy/Vegetable matter	254	44.1
Dust, soil, stone	142	24.7
Unidentified foreign body	88	15.3
Tree, branch, thorn	33	5.7
Insect	27	4.7
Metallic foreign body	17	3.0
Physical violence	12	2.0
Chemical	3	0.5
Total	576	100.0

Table 3: Trauma agents in fungal keratitis patients.

16 different genera (Table 4). Of the 351 isolates sequenced, 175 were identified at species level and the 176 remaining isolates at genus level. *Fusarium* (143; 40.7%) is the most predominant species and followed by *Aspergillus* (91; 25.9%). No yeast isolates was accounted in this study. The DDBJ (DNA Data Bank of Japan) accession numbers for ITS regions of isolates representatives of each fungal species were shown in Table 4.

Treatment

In this study, 1198 of 1687 patients (71%) received medical therapy, 303 patients (18%) underwent therapeutic keratoplasty and the remaining 186 patients were carried out evisceration. Among patients underwent surgical interventions including keratoplasty and evisceration, *Fusarium* spp. were found in 65.6% *Aspergillus* were 32.1% and other species were 2.3%.

Discussion

Due to increasing incidence in past three decades and insignificant responses to antifungal agents [8], fungal keratitis has become one of the leading causes of visual loss in many developing countries, where the large numbers of the population are farmers. Although mycology has undergone remarkable changes by taking full advantages of spectacular developments in molecular biology, and chemistry to improve the understanding of phenotypic and genotypic characteristics of fungi, fungal keratitis remains a diagnostic and therapeutic challenge to ophthalmologist. The difficult matters lie in establishing a clinical diagnosis, isolating the etiologic fungal organisms in the laboratory, and treating the keratitis effectively with topical antifungal agents.

Fungal keratitis is more common in females than in males and often occurs in patients at the middle decades of life. The ratio of female to male patients (1.3 to 1) in this study is completely different from ratios reported in India, China, or Brazil (male to female 1.6:1, 1.5:1 and 6.4:1, respectively) [10-12]. Due to urbanization process, agricultural land is more and more narrowed. Men in rural area have to leave home village to find job in big cities, women at home have to shoulder all men's works. Therefore, this is attributed to the fact that women in rural areas in Vietnam are more involved in agricultural activities, which subsequently increases their vulnerability to fungal keratitis.

We found that the largest proportion of the patients were 40 to 49 years old (27.2%), followed by 50 to 59 (24.6%), which was the same to the scenario in many developing countries such as south India [11], north China [12] and southeast Brazil [10]. This also may be explained by the fact that the subjects ages 40 to 59 in this study are the main force of the manual works, especially agricultural works, and more involved in outdoor activities.

In line with the study done in North China [12], the incidence of fungal keratitis was higher during paddy harvesting and also during the time of year when agricultural work was busier. The peak incidence was seen more prevalent during September to December, which differs from reports in South India noting that fungal keratitis was most common during June to September [13,14]. It can be assumed that the shift from harvest season in this area to the leisure season contributes to sharp increase in the number of cases and the windy, dry weather and monsoon season also affect on the incidence.

Greater number of patients had sought medical help at the National Ophthalmology hospital in duration of symptoms less than one month (87.1%) than those with symptoms longer than one month (12.9%). It is worthy to note that only 38.1% of patients came within one week of onset of symptoms. This rate was much less than that in Madurai, south of India (60.8%) and that in Nepal (43.7%) but greater than that in North China (7.8%) [11,12]. This matter reveals the weakness of primary clinics or secondary medical facilities in accurate diagnosing and treating fungal keratitis. Patients were only referred to the national professional eye hospital from these healthcare systems when empirical therapy was unsuccessful. It also presented the fact that the awareness of the severity of ocular diseases in community was vague. Community health care education issue needs to be taken into account in Vietnam and maybe in other developing countries, as the longer the duration of symptoms, the worse the response of patients to antifungal agents.

Before their initial visit to the National Ophthalmology hospital, 640 patients (93.2%) had received medical therapy. Among them, 551 patients had been administrated combined topical and systemic antibiotics, antibiotic and antifungal, or antibiotic and steroid. This indicates that at the very beginning with or without confirmative diagnosis of agent causing keratitis, antibiotics seem to be the first

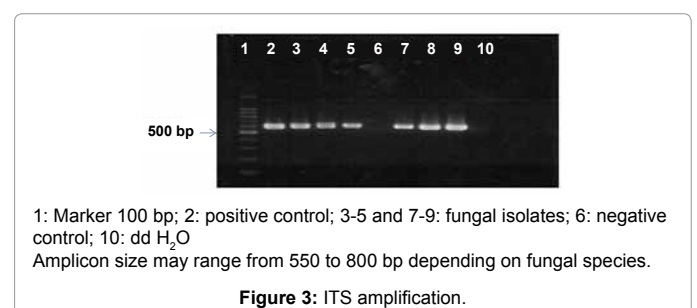


Figure 3: ITS amplification.

Genus	No. (%)	Species (Accession no.)	No. isolates
Fusarium	143 (40.7)	<i>F. solani</i> (AB705146)	31
		<i>F. delphinoides</i> (AB705142)	4
		<i>F. oxysporum</i> (AB705144)	2
		<i>F. subglutinans</i> (AB705156)	5
		<i>Fusarium</i> spp.	101
Aspergillus	91 (25.9)	<i>A. flavus</i> (AB705151)	34
		<i>A. fumigatus</i> (AB705149)	33
		<i>A. peyronelli</i> (AB705149)	2
		<i>A. aculeatinus</i> (AB704778)	1
		<i>A. oryzae</i> (AB705147)	1
		<i>A. terreus</i> (AB704783)	1
		<i>Aspergillus</i> sp.	19
Acremonium	3 (0.9)	<i>Acremonium</i> sp.	3
Scedosporium	1 (0.3)	<i>S. apiospermum</i> (AB705143)	1
Penicillium	9 (2.7)	<i>P. citrinum</i> (AB705148)	6
		<i>Penicillium</i> sp.	3
Eurotium	1 (0.3)	<i>E. rubum</i> (AB705150)	1
Sarocladium	12 (3.4)	<i>S. oryzae</i> (AB705153)	11
		<i>S. attenuatum</i> (AB705154)	1
Curvularia	5 (15.7)	<i>C. lutana</i> (AB705140)	12
		<i>C. affinis</i> (AB705141)	1
		<i>Curvularia</i> sp.	42
Lasiodiplodia	8 (2.3)	<i>L. thebromae</i> (AB704780)	8
Phoma	4 (1.1)	<i>P. putaminum</i> (AB704776)	4
Bipolaris	4 (1.1)	<i>Bipolaris</i> sp.	4
Alternaria	2 (0.6)	<i>Alternaria</i> sp.	2
Cladosporium	1 (0.3)	<i>Cladosporium</i> sp.	1
		<i>C. gloeosporioides</i> (AB704781)	10
Colletotrichum	16 (3.8)	<i>C. dematium</i> (AB705152)	1
		<i>C. truncatum</i> (AB704777)	1
		<i>Colletotrichum</i> sp.	1
Myrothecium	2 (0.6)	<i>M. inudatum</i> (AB704780)	2
Coprinopsis	2 (0.6)	<i>C. cinerea</i> (AB705155)	2
	351 (100)		351

Table 4: Fungal isolates from fungal keratitis.

miracle drugs of the general physicians and ophthalmologists at the primary clinics or secondary medical facilities in Vietnam. They also have tendency of prescribing more than one drug for surrounded treatment and referring patients to the national professional eye hospital when patients had experienced their empirical treatment fails. Ten patients had used corticosteroid, a kind of medication that could weaken the defense mechanism of the cornea, thereby favoring the fungal growth and progression of the disease [8]. Long-term use of antibiotics also is believed to compromise the cornea. The fact that is worthy to worry is the inappropriate and abuse antibiotic use in Vietnam. Even though prescriptions are legally required, antibiotic and many other drugs can be purchased directly by consumers at pharmacies or drug outlets in Vietnam. Additionally, self-diagnosis is not very accurate, but self-treatment is very common. This context is also seen in the present study. There were 83 patients either self-buying drugs without physician's prescription or getting empirical treatment from untrained village healers. Therefore, it is very important to reach a definite diagnosis and start proper antifungal therapy for fungal keratitis patients to save patient vision and minimize corneal scar.

Corneal trauma is the most frequent and major associated condition for fungal keratitis. In fact, history of corneal trauma, particularly with vegetable or dust, soil matters. Contact lens wear was reported one of the major associated conditions in industrial countries in many studies [15,16] but there was no case of contact lens wear in our study. The

possible reason is that the habit of wearing contact lens is not common in Vietnamese, particularly in the poor farmers.

363 (52.8%) of 687 patients diagnosed fungal keratitis, were culture positive, the remaining 324 (47.2%) were only smear positive. The proportion of culture positive in this study is lower than that in the study carried out in West Bengal, India [1]. The possibility of negative culture results of fungal infection patients might result from either the very small amount of specimen collected or unculturable causative agents. Thirteen scrapings from negative culture patients were selected randomly for PCR amplification directly without culture. After analyzing ITS regions, five of thirteen samples were identified as unculturable fungi and eight were filamentous fungi (data not shown). No case of yeast pathogen was found in this study. Twelve samples that culture grew were either negative by PCR or positive by PCR but poor sequences. This could be due to either sequence variation of ITS regions of these fungal isolates. Finally, only 351 ITS region sequences were obtained and analyzed. The ITS sequence analysis showed that *Fusarium* spp. was the predominant species, similar to the reported from South Florida and Ghana [13], southeast Brazil [10], north China [12], Malaysia [17] where the climate is warm and humid. This is contrast to most reports of *Aspergillus* spp. from north India [18] and *Candida* from some developed countries [19,20]. Most filamentous fungi associated with corneal keratitis in the tropics are found widely within the environment. In Vietnam, the unavailability of commercial kit for identification filamentous fungi limited diagnostic ability of clinical laboratories. Many laboratories could only either differentiate mould from yeast or identify a few typical filamentous genera. Analysis of ITS sequences can be identified most of fungal isolates at species level but limited in identification of *Fusarium* species. Further study using other specific makers for identification of *Fusarium* isolates at species level will be done.

Surgical intervention in the form of therapeutic keratoplasty is an important mode of fungal keratitis management. However, the numbers of patients needed therapeutic keratoplasty were much greater than those of patients underwent keratoplasty. Most of patients in this study got keratoplasty were in cases of need to get immediate keratoplasty. The reason of this context is that the source of corneal tissue from donors is limited in Vietnam and always insufficiently meets the need of corneal transplantation. Many patients having indication of keratoplasty have to be in the waiting list for long time until receiving donors' corneas.

In conclusion, the etiological and epidemiological pattern of corneal ulceration varies significantly with patient population, health of the cornea, geographic region, climates and also tends to vary overtime. Hence, for the effective prevention and treatment of fungal keratitis, it is important for ophthalmologists to be aware of regional epidemiological features, risk factors, and etiologic data concerning this disease.

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