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Fungal keratitis: Pathogenesis, diagnosis and prevention

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ABSTRACT

As a kind of serious, potentially sight-threatening corneal infections with poor prognosis, fungal keratitis can bring a heavy economic burden to patients and seriously affect the quality of life, especially those in developing countries where fungal keratitis is more prevalent. Typical clinical features include immune rings, satellite lesions, pseudopods, hypha moss, hypopyon and endothelial plaques. The ideal therapeutic effects could not be achieved by current treatments for many reasons. Therefore, under the current status, understanding the pathogenesis, early diagnosis and prevention strategies might be of great importance. Here, in this review, we discuss the recent progresses that may advance our understanding of pathogenesis, early diagnosis and prevention of fungal keratitis.

1. Introduction

Fungal keratitis has a high frequency (about 1,000,000 new corneal infections per annum) and a high blindness rate, accounting for 1–45% in infectious keratitis [1,2]. The incidence of fungal keratitis is higher in developing countries, compared with that in developed countries [3]. Compared with other infectious keratitis, fungal keratitis has a poor prognosis, due to not only the lack of effective treatment drugs and methods, but also that fungi are different from other pathogens in pathogenesis. Prevention, early diagnosis and early treatment of fungal keratitis can undoubtedly improve the curative effect and the prognosis of patients, and reduce the blindness rate. Therefore, it is of great clinical significance and social value to study the preventive methods, pathogenesis and diagnose of fungal keratitis, which will be discussed in this review, in the hope that these manners can be applied in clinical practice.

2. Pathogenesis of fungal keratitis

2.1. Pathogen and risk factors

The common pathogenic fungi causing keratitis are species of *Aspergillus, Fusarium, Candida, Curvularia,* and *Penicillium*, among which *Fusarium* and *Aspergillus* were the main ones [4–7]. Infections caused by *Fonsecaea pedrosoi* [8], *Lasiodiplodia theobromae* [9], *Cylindrocarpon species* [10], *Scedosporium prolificans* [11], *Metarhizium anisopliae* [12], *Paecilomyces species* [13], and *Pythium insidiosum* [14] have been less reported. The prevalence of fungal pathogens is different in different genders. The reported morbidity of fungal keratitis in men was higher than that in women, with a ratio of 1.6:1 [15,16]. The reason may be that the proportion of men engaged in agricultural and high-risk works is higher, while these works carry a higher risk of ocular traumatic infection. The prevalence of fungal keratitis may vary greatly in terms of species and regions. The prevalence of *Candida* was 60.6% in London [17] and 32.7% in Melbourne [18]. *Acremonium* spp. are the main pathogens of fungal keratitis in Paraguay [19]. Fungal keratitis is common in rural areas of northwest Rajasthan, mainly in men with low socio-economic status (aged 21 to 40, farm or factory workers), and the most common cause is *Aspergillus flavus* [20].

The risk factors for fungal keratitis are as follows: 1. history of plant trauma or trauma caused by objects contaminated with soil [21]; 2. contact lenses [22,23]; 3. ocular surface disease; 4. lacrimal duct occlusion [24]; 5. fungal skin infections (including onychomycosis) [25]; 6. long-term use of antibiotics or steroids locally or systemically [17]; 7. other factors include history of eye surgery, herpes simplex virus keratitis, eyelid abnormalities, etc [5,26,27].

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2.2. Pathogenesis

Various aspects of pathogenesis of fungal keratitis have been published in the last several years. They describe the conditions required for fungal infections, the pathogenesis of fungal keratitis in adhesion, invasiveness, morphogenesis, toxigenicity, fungal survival and replication within macrophages. Fungal infections require a) to survive and grow at cornea; Human central corneal temperature is 32.6 ± 0.70 °C [28], which was suitable for the growth and toxigenecity of *Fusarium* and *Aspergillus*, two of the most common pathogens of fungal keratitis.; b) the ability to penetrate internal tissues in spite of host defenses; c) the ability to digest and absorb nutrients in host; d) the ability to resist host immune system [29]. Each step of fungal pathogenesis is closely linked. We further review the recently published articles, providing the relevant mechanisms explored in the past few years. To make things simple, the pathogenesis is divided into two parts: fungal growth and corneal inflammation.

2.2.1. Fungal growth

To initiate infections, fungal cells must adhere to surfaces of tissues, which is mediated by interactions between adhesins and epithelial surfaces [30]. Fungi produce a variety of surface proteins (mainly mannoproteins) to contribute to the adhesion to the corneal epithelium, which has potential fungal binding sites such as laminins, fibronectins, and collagens [30]. Upon the contact between fungi and host, fungi can be recognized by pattern recognition receptors (PRRs) expressed on host epithelial and immune cells. PPRs include Toll-like receptors (TLRs, including TLR2 and TLR4), C-type Lectin receptors (CLRs, including Dectin-1, Dectin-2 and Mincle (Macrophage-inducible C-type lectin)), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) which don't directly contribute to fungal recognition [31]. Dectin-1 recognizes β-glucan in fungal cell wall, while Dectin-2 and Mincle recognize mannan of cell wall [31]. *Aspergillus fumigatus* encounter during fungal keratitis could be sensed by TLR2, TLR4, Dectin-1, Dectin-2 and Mincle, and the crosstalk between these receptors can be found [5,31–33]. Activation of TLRs in corneal epithelium induce production of CXC chemokines and recruitment of neutrophils, which account for more than 90% of the infiltrating cells and are the predominant source of mature interleukin-1β (IL-1β) and acidic mammalian chitinase (AMCase) in corneas, both of which can inhibit the hyphal growth [34–36]. The increased expression and the activation of these PPRs in response to *A. fumigatus* challenge result in increased secretion of inflammatory cytokines, such as IL-1β, IL-6, IL-8, IL-17 and IL-23 in human corneal epithelial cells and neutrophils [5,34,37]. The elevated levels of IL-1β, TLR4, Dectin-1 and LOX-1 can increase the production of ROS, which facilitates the fungal killing [5,38,39].

The host defense mediated by epithelial cells, macrophages, neutrophils and dendric cells, could be overcome by fungal pathogens. The binding of β-glucan on fungal cell wall to Dectin-1 and CD18 can activate NOX, which is required by neutrophils to generate ROS that suppress hyphal growth of filamentous fungi (such as *A. fumagitus*, *A. flavus* and *F. oxysporum*) in keratitis, while the antioxidant resistance of A. *fumigatus* to human neutrophil killing in fungal keratitis depends on SOD and transcription factor Yap1, rather than catalase and gliotoxin which have been shown to inhibit NOX in other tissues [40]. Encounter of *A. fumigatus* with oxidative stress can activate Yap1, resulting the upregulation of gene products with anti-oxidative properties, (such as thioredoxin) which are required for impaired fungal killing by neutrophils in fungal keratitis [40]. During oxidative stress, the transcription factor Skn7, which regulates the expression of protein phosphatase PhzA, also contributes to the resistance to host defense and PhzA mutant of *A. fumigatus* showed decreased pathogenicity, as evidenced by the decreased opacity, fungal burden and hyphae in corneal ulcers in mice [41].

Although antigens and allergens on the surfaces of fungal spores are targets of the immune system, hydrophobin, a highly insoluble protein complex present in the outermost layer of fungal spores, can prevent the recognition by immuno system [42,43]. RodA, the first found hydrophobin, can inhibit the recognition of conidia by Dectin-1 and Dectin-2, thereby avoiding phagocytosis by macrophages [44]. In the absence of RodA protein, the ligands on the surface of the spore can be unmasked and recognized, leading to the phagocytosis and killing of spores by macrophages. Developing antifungal drugs acting on hydrophobins may be a potential strategy in the treatment of fungal keratitis [45].

Fungal invasiveness is facilitated by their capacity to produce enzymes that degrade physical barriers and antimicrobial proteins. For example, mycotoxins from *Fusarium* species can inhibit immunity and break down tissues. Corneal epithelial cells can be destroyed by some cytosolic proteins and peptide toxins produced by fungi [46]. Besides, mycotoxins can also be produced to promote the fungal survival in host. The protease and phospholipase activities can be detected in many strains (such as *A. flavus* and *F. solani*) isolated from human eyes [47]. A number of studies have found that proteases play an important role in fungal keratitis, as they can cause corneal ulceration [48]. While the serine proteinase activity of *A. flavus* and *F. solani* can trigger corneal cell activation [49]. Lectins from *Cephalosporium curvulum* (CSL) and *Aspergillus oryzae* (AOL) can bind to TLR2 and TLR4 of corneal surface, inhibit host cell growth and destroy the integrity of the epithelial cells [50].

2.2.2. Inflammation

The initial fungal contact during corneal infections increases the expression of PPRs, which promote the production of pro-inflammatory cytokines through MyD88/NF-kB signaling or Syk/PKCδ/Card9-Bcl-10-MALT1/NF-kB signaling, as well as the recruitment of neutrophils [5,31,39,51]. Although neutrophils are the mainstays to fight invading fungal pathogens, they can also cause serious inflammatory damages to cornea (opacification) [34].

During *A. fumigatus* keratitis, the increased expression of LOX-1 and TLR4 can lead to elevated IL-1β production, which requires caspase-11 for maturation and stimulates ROS production in neutrophils, and ROS in turn induce the production of IL-1β and promotes inflammatory cell recruitment [34,38]. Although ROS is essential for fungal killing of neutrophils in keratitis, it can also cause damages to surrounding cells and organelles including mitochondria, inducing and amplifying inflammation [36,38].

In fungal keratitis, the levels of pro-inflammatory IL-1β, IL-6, IL-8, IL-17, IL-23 and IFN-γ in aqueous humor were significantly higher than those in the non-keratitis control group [36,37,52,53]. Among these cytokines, IL-1β was considered as a marker reflecting the severity of inflammation, while the production of IL-1β, IL-6 and IL-23 can cause Th17 cell differentiation, promoting the secretion of IL-17 and IL-22 [31,37,38]. IL-6 and IL-23 are required for IL-17 production in neutrophils and IL-17 can also induce ROS production [36,37].

Due to the significant role of IL-1β in fungal keratitis, it is considered as a target for treating fungal keratitis. In IL-1β $^{-/-}$ mice, corneal opacification caused by *A. fumigatus* infection was decreased significantly, as compared to controls [34]. Decreasing IL-1β maturation through caspase-1 suppression by wedelolactone, in combination with antifungal treatment, could also reduce the corneal opacification in mice with *A. fumigatus* keratitis [54]. This may lead to an interesting envisage: would a compound with both antifungal and anti-inflammatory activities, such as dioscin, have similar or better effects on treating fungal keratitis [55,56]?

3. Diagnosis

The incidence of keratitis can be reduced by early identification of keratitis pathogens, which provides a prerequisite for early treatment of keratitis. The history of trauma and contact lens wearing, as well as

other clinical factors, need to be considered before diagnosis. A definitive diagnosis is based on clinical manifestations and supported by laboratory diagnostic methods. Laboratory diagnostic methods include clinical specimen culture and staining, microscopic observations, molecular detections and so on.

3.1. Traditional diagnostic methods

The traditional diagnostic method is mainly to stain and culture the clinical specimens after scraping the tissue from the cornea. At present, the diagnosis of fungal keratitis mainly relies on fungal culture, and it is more accurate to identify the fungal pathogen. However, there are several disadvantages. For example, the risk of complications caused by invasive tissue sampling, and the longer time required to identify pathogens (cultivation time is generally 1–2 weeks, sometimes even more than one month) [57]. The diagnosis speed and accuracy are greatly limited, leading to the difficulty to carry out appropriate antifungal treatment in time [6,58]. The rapid staining method is a direct detection method, which is relatively simple, fast and inexpensive. KOH is one of the most commonly used methods for direct microscopic detection of fungal pathogens. Although both the specificity and sensitivity in fungal keratitis were reported to be higher than 98%, the degree of sensitivity may vary largely among different groups, while individual experience of microscopic examination, corneal ulcer size and the amount of sampling material can affect the sensitivity of this method [59–61].

3.2. Novel diagnostic methods

3.2.1. In vivo confocal microscopy (IVCM)

IVCM is a new type of equipment for early diagnosis and guiding the treatment of fungal corneal ulcer in recent years. It has been widely used in clinical practice to observe the morphology of fungal hyphae dynamically in the cornea (Fig. 1) and has a wide range of applications in fungal eye infections [62–64]. Based on confocal microscopy, the diagnostic method of IVCM in fungal keratitis has been improved in different ways.

Using a series of pinhole gratings, IVCM can be employed to observe fungal hyphae directly in the cornea, with a resolution of 1 μm [65]. The morphological features of different species, such as hyphal branching angles along spore formation (accidental sporulation) or bipartites of hyphae, can be distinguished by IVCM [62]. The mycelium length of both *Aspergillus* and *Fusarium* is 200–400 μm, the branching angle of *Aspergillus* is 45°, while that of *Fusarium* is 90°. Yeast is oval in shape and produces pseudohyphae, which are 10–40 μm in length and 5–10 μm in width. It is worth noting that the fungus needs to be distinguished from the basal corneal epithelial nerves, and these can be distinguished by differences in diameter. The basal corneal epithelial nerves are much larger than fungal hyphae, for example, the diameter

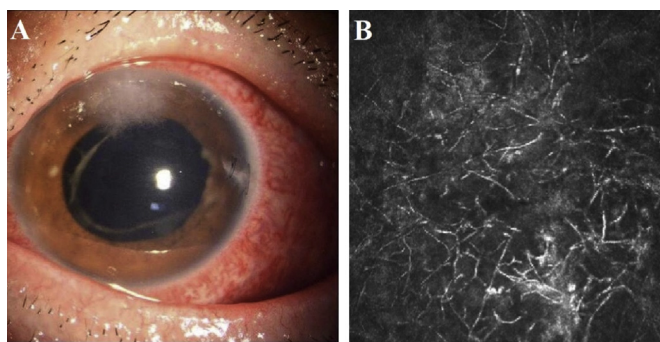

Fig. 1. Keratitis due to *Aspergillus terreus*. A. Slit-lamp picture of the cornea of the left eye from a 50-year patient. B. IVCM photograph of fungal hyphae, which are high reflective filaments.

of nerve is 5–10 times larger than that of the *Aspergillus* mycelium. However, it is almost impossible to distinguish *Fusarium* from *Aspergillus* by IVCM, because the fungal branches are not common, in spite of the big differences in branching angle. Therefore, cultivation is still the key method to identify the pathogens of fungal keratitis [62]. Compared with traditional smear microscopy, IVCM is not only non-invasive, but also consistent with traditional methods in sensitivity and specificity [66], and even better in some studies [67–69]. Meanwhile, IVCM can be used for the detection of the depth of fungal infection, acting as an important diagnostic tool for fungal keratitis. Although the method has many advantages, there are still some limitations, such as the need for a skilled operator or an experienced viewer; the cooperation of patients during the observation; the heavy influence that may be imposed by scars [65].

3.2.2. Tomographic imaging probe

Caspofungin belongs to the echinocandin class of antifungal drug and can form a complex with the fungus-specific enzyme β-1,3-D-glucan synthase. Therefore, caspofungin is highly specific to fungi [70]. The modification of functional site ethylenediamine by BODIPY, doesn't affect the high efficiency of targeting for fungi, while the addition of fluorescence tag 7-Amino-9H-(1,3-dichloro-9,9- Dimethylacridin-2-One) (1,3- dichloro-9,9- Dimethylacridin-2-One) (DDAO) on the residue of 3-hydroxyornithine, makes this caspofungin derivative a specific probe for fungal detection. Using near-infrared (NIR) fluorescence imaging, the fungal cells binding fluorescent probe can be seen [71]. If the drug is coated with liposomes, it can promote the penetration of the probe (L-caspofungin DDAO) into the cornea better [72]. Min Hee Lee et al. found [73] that fungi can be detected *in vitro* using the L-caspofungin DDAO probe, which can specifically bind to corneal-infecting fungi in a mouse model of fungal keratitis *in vivo*. Using this method, the fungal can be detected by confocal microscopy in 5 min after local application of the probe. However, this kind of detection has only been performed in *Aspergillus* keratitis and *Fusarium* keratitis. Further research and more strains are needed to extrapolate the conclusion in other fungi, with the ultimate goals of early application in the clinic to promote the early diagnosis of fungal keratitis and appropriate treatment of corneal fungal infections.

3.2.3. Two-photon microscopy (TPM)

TPM is a three-dimensional microscopic technique based on nonlinear two-photon fluorescence [74], through which cells and corneal collagen were imaged separately by the intrinsic contrast of autofluorescence (AF) and second harmonic generation (SHG). Compared with confocal microscopy, it reduces both light damage and the scattering of infrared light as it penetrates living tissues to enable imaging of deeper structures [75–77]. Compared to reflectance confocal microscopy (RCM), TPM can be used to observe deeper tissues with longer-wavelength light and it is less affected by corneal opacity [78–81], but the imaging time is longer. Moxifloxacin can penetrate tissues, emit fluorescence and aggregate in cells, so moxifloxacin is used as a cell marker for TPM imaging. Jun Ho Lee et al. studied the TPM imaging of *in vitro* fungal keratitis in rabbit corneas caused by *A. fumigatus* and *C. albicans* [82]. They found that the fluorescence signal of fungal cells treated with moxifloxacin in the rabbit cornea was clearly visible and increased 10-fold [82]. Therefore, the labeling of moxifloxacin and TPM can be used for the detection of fungal keratitis, with an increased speed of imaging.

3.3. Molecular detection

Traditional smears, as well as culture and morphology-based fungal identification, do not always provide enough resolution for identifying fungal species, and under these conditions, molecular detection would perform well and shorten the time consumed to produce reliable results.

rDNA sequences, which encode rRNAs of the small and large

Fig. 2. The sequence located between the 3′ end of the 18S rRNA gene and the 5′ end of the 28S rRNA gene is called an internal transcribed spacer (ITS). The sequence located between the 3′ end of the 25S rRNA gene and the 5′ end of the 18S rRNA gene is called an intergenic spacer (IGS). Various rRNA genes are separated by some non-transcribed compartments.

ribosomal subunits, are most conserved nucleic acid sequences in multiple copies, making them suitable targets for diagnosis [83]. Eukaryotes contain four subunits, including 18S, 5.8S, 28S and 5S, which are connected head to tail on chromosomes (Fig. 2), can provide a classification basis for lower ranks (such as species, families, genera, and partial species) [84–87]. The internal transcribed spacer (ITS) and the intergenic spacer (IGS) can provide a classification basis for higher ranks (such as species, subspecies, varieties, and even strains) [88,89].

3.3.1. PCR (polymerase chain reaction)

The diagnosis by PCR is to amplify the fungal DNA sequences obtained from corneal lesions to identify the fungal species. PCR is a fast and highly sensitive method for the diagnosis of fungal keratitis, even in some cases with negative results obtained from culture [90]. PCR has different sensitivities in different flora, for example, the amount of DNA required for *Fusarium* is 10 fg/μL, *A. flavus* is 1 pg/μL, and *A. fumigatus* is 300 pg/μL [91]. However, PCR still has many disadvantages, such as cross-contamination with conjunctive pathogen flora, over-diagnosis by non-pathogenic bacteria, and the requirement for suspicion of targeted pathogens due to the lack of a panfungal discrimination [90,92]. These disadvantages, along with the low amount of fungal DNA in clinical samples (compared to bacterial and viral pathogens), has promoted the pursue for strategies to improve the sensitivity of PCR detection. Several types of PCR, including real-time quantitative PCR (q-PCR), multiplex PCR, loop-mediated isothermal amplification (LAMP), etc., are listed in Table 1, along with their main strengths and weaknesses.

3.3.2. DNA sequencing

As the gold standard for microorganism identification, DNA sequencing can not only distinguish species more rapidly, but also identify clinically rare species [7,102]. Furthermore, it can also help predict/identify antifungal drug sensitivity and treatment outcomes, thus benefiting the treatment (through stratified therapies according to different strains) and improving the prognosis [102,103]. However, it is time-consuming and requires skilled technicians and expensive laboratory equipment. Therefore, it is now mostly used for research and not used in clinical practice.

ITS is a universal DNA barcode marker for fungi. At present, these fragments are used to identify the fungi of several genera to the species, such as in the genus *Trichosporon*, *Candida* and *Aspergillus* [104], even rare filamentous fungi like *Pythium insidiosum* [14]. ITS regions analysis has high sensitivity and specificity in the identification of the phylogenetically similar species [105]. ITS1 is more variable than ITS2, presumably allowing better discrimination among species, however, this is in debate [106]. Although species-level identification can be reached by ITS sequence, high differences in length and sequence weaken the distinguishing power for classification at high taxonomic ranks [106].

The 18S nuclear ribosomal small subunit rRNA gene (SSU) is commonly used in phylogenetics, and it has fewer hypervariable domains in fungi. The 28S nuclear ribosomal large subunit rRNA gene (LSU) sometimes discriminates species on its own or combined with ITS [106]. For yeasts, the D1/D2 region of LSU was adopted for characterizing species long before the concept of DNA barcoding was promoted [107]. *Trametes betulina*, which can cause secondary fungal keratitis, as well as *Scytalidium cuboideum* and *Arthropsis hispanica,* can be identified by sequencing the ITS and D1/D2 regions [102,108]. D2 is less able to distinguish species of fungi than ITS [109]. Single gene sequence of D1/D2 has limitations for identifying hybrids [107]. ITS and D1/D2 regions are not always amenable to amplification and direct sequencing, as they sometimes contain large introns, heterogenic regions and/or poly A/T regions, which prevent PCR amplification and/or interfere with sequencing [110].

The IGS region is more variable than other existing loci of interest, allowing the separation of clinical *Cryptococcus neoformans* isolates, as well as strain-typing of *Pichia anomalam* (IGS1), *Kluyveromyces marxianus* and *Kluyveromyces lactis* (IGS2) [107]. The interspecies variability of IGS sequences is useful for epidemiological analysis, however, it is not suitable for discriminating some fungal pathogens, such as *Malassezia globosa* and *Malassezia restricta* [105].

Table 1
Strengths and weaknesses of different platforms.

Methodology	Strengths	Weaknesses	References
Traditional PCR	High sensitivity and specificity	Cross-contamination Over-diagnosis by non-pathogenic bacteria	[90,93,94]
Multiplex PCR	Good specificity High separation rate	False negative results	[95–97]
q-PCR	Quantitative Low risk of contamination	Non-specific binding	[98,99]
LAMP	High specificity, sensitivity and efficiency Low requirements and price	Contamination-prone Amplification of short-stranded DNA with small fragments	[99–101]

3.4. Image-based diagnosis

3.4.1. Image-based automatic hyphae detection

Based on the images obtained by confocal microscopy for automatic recognition, this method employs adaptive robust binary pattern (ARBP) analysis and support vector machine (SVM) models, coupled with line segment detector (LSD), for hyphal quantification and classification. Data collected from 79 patients (56 patients with monocular fungal keratitis and 23 patients with bacterial keratitis) to analyze the consistency of clinical symptoms and mycelial density revealed that: the accuracy of this image-based technique was superior to corneal smears, with a sensitivity of 89.29% and a specificity of 95.65% [111]. However, there are several problems with this method. It is well known that confocal microscopy is superior to corneal smears in diagnosis. Whether this method of automatic identification of hyphae is better than confocal microscopy still needs further verification. The advantage is obvious: this auto-detection does not rely on technicians and viewers, the training of whom need much more time and resources. It can help young doctors with insufficient experience, by providing timely, accurate, objective and quantitative evaluation criteria for fungal keratitis [111].

3.4.2. Smartphones-based imaging and diagnosis

Imaging technologies are improving with the increasing prevalence of smartphones. It is possible to diagnose fungal keratitis on the spot rapidly, economically and reliably by smartphones, which have simple lens systems, microsensors, micro-processing and displaying systems.

Combined with fluorescent labeling, smartphones can help to detect microbes and parasites, even subcellular proteins and nucleic acids *in vitro* [112–114]. Agarwal et al. recently reported the use of smartphone-based digital imaging in the diagnosis of fungal keratitis and follow-up surveys [115]. The patient's lesions can be photographed and enlarged on displays for the fungal hyphae detection by using a smartphone, combined with a microscope and integrated light-emitting diode assembly. Compared with traditional detection of corneal scraping and staining, smartphone-based imaging has great portability and accessibility, making the instant diagnosis more easy, especially in a distant follow-up survey when the experienced doctors are far away, due to the convenient network transmission [115]. Also, smartphones can be the carriers of artificial intelligence (AI), while AI-assisted diagnosis has been proposed and studied for many years [116,117]. At present, the application of this technology is still in its infancy, and undoubtedly more and more focus will be concentrated in this field because of the advantages it harbors.

4. Prevention

The management of fungal keratitis is a major challenge, while delayed diagnosis or untimely treatment or malpractice can allow fungal growth. Fungal infections are difficult to eliminate, once fungi enter the Descemet membrane (DM). Fungi will gradually penetrate the elastic layer of the cornea and eventually cause endophthalmitis, which may lead to the enucleation of eyeball. The low penetration of drugs in eyes/tissues and the ease of developing antifungal resistance, are the two major difficulties with antifungal drug therapies, making both drugs and surgery unsatisfying. Antifungal drug resistance may result from reduced intracellular drug accumulation, changes in drug targets, changes in membrane sterol synthesis pathways, alterations in fungal cell wall components, biofilm formation, and morphogenesis of fungi [125–127]. To improve the poor penetrability of local drugs (such as natamycin and voriconazole) towards corneal epithelium to gain a satisfactory therapeutic efficacy, many carrier molecules have been devised. At present, as the main surgical treatment, penetrating keratoplasty (PKP) is confronted with a high relapse rate, more surgical complications, insufficient donors and so on. Due to the lack of mature and standardized treatment regimes, the efficacy of Rose Bengal photodynamic therapy still needs to be further justified. The failure of treatment will lead to corneal ulcer, the cure of which is expensive, higher than the average monthly salary of the population involved, producing a huge economic burden on patients and their families. Even if the ulcer heals, visual recovery will not be optimal, and it is estimated that at least one third of the patients eventually suffer from impaired eyesight [128]. Considering the prevalence of fungal infections in the worldwide, the unsuccessful outcomes despite of antifungal therapy, the scarcity of antifungal drugs available, as well as the heavy burden fungal keratitis may bring, it is of great importance to prevent corneal fungal infections.

4.1. Vaccine

In vaccine development, the dose, growth phase (live vaccine does not need to consider growth phase), vaccination site, vaccination route need to be taken into account [129]. Over these years, many formulations have been tested as potential vaccines in the field of fungal vaccine research. At present, there are many components of fungal vaccine formulations, such as killed organisms, attenuated strains of organisms, specific proteins and other cellular components from fungal pathogens. Despite a lot of endeavors, no fungal vaccine has been approved for clinical use.

In early studies, most vaccines were directed at a single specific fungus without evaluating cross-reactions [130]. Currently, antibody-mediated protective effects against candidiasis, cryptococcosis and aspergillosis are induced by conjugated laminarin (β-glucan) [131]. Proteins from *Saccharomyces cerevisiae* can cross-react with sera infected by other fungi while homologous proteins in cell wall proteins of different fungi were found by proteomic technologies. All these proved that a pan-fungal vaccine is possible [132]. At present, cross-protection against some fungal infections by xenogeneic fungal immunization has become an important way to develop fungal vaccines. Heat-killed yeast (HKY) of *S. cerevisiae* could induce spleen and LN cells to proliferate and produce more IFN-γ, IL-6 and IL-17 in mice. HKY can also induce Th1 immune response and the production of antibodies against β-glucan and mannan to protect mice from fungal invasion [133]. These mechanisms enable HKY to induce protecting effects against fungal infections caused by *Coccidioides*, *Aspergillus*, *Coccidia*, *Candida*, *Cryptococcus* and *Rhizopus*, which further confirmed the plausibility of developing pan-fungal vaccines [133].

Poly-N-acetyl-β-(1–6)-glucosamine (PNAG) is a polysaccharide expressed by many microorganisms, such as Gram-positive, Gram-negative bacteria, fungi and so on. The human body can produce PNAG antibodies, but it can't promote phagocytosis of phagocytes due to the lack of deposited opsonin, so PNAG antibodies usually have no protective effect on fungal infections [134]. In *A. flavus* and *F. solani*, polyclonal antibodies and human IgG1 monoclonal antibodies can produce high levels of opsonic killing mediated by PNAG [135]. This protective mechanism is related to IL-17 and IL-22, which can induce the immune response of T cells and neutrophils, thereby reducing the fungal load in the corneas of mice [136,137]. Local injection of antibodies after infection can also be used to treat fungal keratitis. Therefore, the active or passive vaccination of PNAG antibody can be used not only for the prevention of infections in high-risk population, but also for the treatment of infection after corneal injury.

Liposomes-encapsulated mannan extracts from *C. albicans* stimulate the production of antibodies protective against candidiasis in mice [138]. A vaccine that conjugated with protein laminaran (algal glucan) linked to a carrier protein diphtheria toxoid, have a good killing effect on fungi containing glucan in cell wall [139]. There have been many studies on vaccines for fungal disease antibodies in other tissues such as the vagina and oral cavity, which may provide suggestions for developing fungal keratitis vaccine.

4.2. Commensal flora

Composed of bacteria, viruses and fungi, commensal flora inhabits the surfaces of the mucosal barrier, providing a physical barrier against the invasion of pathogens. Commensal flora plays an important role in maintaining the immune homeostasis of local tissues such as gastrointestinal tract, skin, urogenital tract, oral cavity and respiratory tract [140,141]. The use of local antibiotics in the eyes makes the eyes susceptible to infection, suggesting the existence of commensal flora on the surfaces of the eyes, which was further confirmed by St. Leger AJ et al. [142]. *Corynebacterium mastitidis* that colonize the ocular surface, can induce γδ T cells to produce IL-17 in the eye mucosa. IL-17 released into tears can induce antimicrobial peptides such as defensin to eliminate invading bacteria and fungi [143]. This commensal bacterium increases the defense of host to resist the infections of invasive *C. albicans* and *Pseudomonas aeruginosa* on the ocular surfaces. This discovery implies the potential use of probiotics in the prevention and treatment of fungal diseases.

Probiotics were defined as living microbial feed supplements, which can improve the intestinal microbial balance of host animals [144]. They can inhibit the growth of pathogens, regulate the immune response of the human body and prevent the occurrence of diseases. *L. plantarum* CMPG5300 can co-aggregate with *C. albicans*, inhibiting the adhesion of *C. albicans* to mucosal surfaces [145]. In closely related taxa *L. rhamnosus*, *L. casei* and *L. paracasei*, the major peptideoglycan hydrolase, Msp1, is the key effector molecule to inhibit hyphal growth of *C. albicans*. Through breaking down chitin, the main polymer in the cell wall of *C. albicans*, Msp1 contributes to the prevention of candidiasis

[146]. Probiotics, such as *L. rhamnosus*, *L. acidophilus*, *L. pyogenes*, *L. casei* GG and Bifidobacterium, can protect mice from candidiasis by eliciting protective immune and non-immune responses [147–149]. The beneficial effects of probiotics have been studied in gastrointestinal tract, vagina and respiratory system, while the mucosa of conjunctiva is similar to those of gastrointestinal tract and vagina. This may further facilitate researches into the potential use of probiotics in ocular surface for diseases prevention.

4.3. Antimicrobial peptides (AMPs)

AMPs, also known as host defense peptides, are naturally produced, small, cationic, amphiphilic peptides ranging in length from 12 to 50 amino acids [150]. They are present on the surfaces of the eyes and in tears. There are various types of AMPs, and more than 500 AMPs have been reported, including some larger molecules (such as RNases and S100A proteins), small peptides α and β defensins in human cationic antibacterial protein (CAP) 18, and α 37 amino acids. Some of them have significant bactericidal activity, such as lysozyme and peptidoglycan recognition protein. Cationic peptides as antibacterial agents have several distinct advantages. They can kill a broad spectrum of microorganisms, including bacteria, viruses, fungi and parasites, and they can be easily synthesized with low risk of drug resistance [151]. Due to the obvious advantages of AMPs abovementioned, some AMPs are currently in clinical tests to prevent and treat infections caused by fungi, drug-resistant super bacteria and human immunodeficiency virus, as well as to treat cancers [152]. AMPs are also involved in the proliferation of tissue cells, wound healing and chemotaxis, among which AMPs have different action mechanisms [153,154]. The cations carried by AMPs can bind to the anion surface of the bacterial plasma membrane, causing the perforation of cell membrane and subsequently microbial death [155,156]. AMPs also prevent microbial adhesion to and access into host cells. In addition, fungal cell wall can be digested by lysozyme, an important AMP in animals [151,157]. The antimicrobial potential of AMPs may vary largely among different parts of the same organism, as well as among different species. In tears, the increased levels of AMPs may reflect the presence of microbial pathogens in eyes [158]. In the pathogenesis of ocular fungal infection, AMPs are secreted on the surfaces of eyeballs, to further block the invasion of fungal pathogens and to prevent diseases. AMPs can be used not only in the early stages of fungal infections in eyes, but also in the treatment of fungal keratitis. The *in vivo* therapeutic effects of synthetic β-sheet forming peptide (IKIK) NH2 and (IRIK) NH2 were equivalent to that of amphotericin B (AmB) in treating fungal keratitis [159]. Ocular tissue-related antimicrobial peptides include S100A, CXCL10, Chi3L1, clavanin A and so on.

4.3.1. S100 proteins

The S100 proteins are a group of 10–12 kDa small amino acid proteins that participate in important cellular activities, such as calcium homeostasis, cellular stress, cell division and differentiation, by binding to different proteins including TLR4, and some of them are involved in antifungal and immune responses of cornea [160]. As a type of S100 protein, S100A is widely present in the eye. Produced by neutrophils, monocytes and epithelial cells, the heterocomplex of S100A8 and S100A9 shows strong antifungal activities *in vitro* and regulate the leukocyte trafficking [161]. In addition, S100A8-targeting antibody has also demonstrated promising results in the management of corneal neovascularization [162]. The expression of S100B in corneal epithelial cells both *in vivo* and *in vitro* can be increased by inactive *A. fumigatus* stimulation, suggesting the participation of S100B in the innate immune response and in the resistance of cornea to fungal infections [163]. Subconjunctival injection of S100A8 or CXCL10 can significantly reduce the incidence of microbial keratitis, with pathogen load as an indicator of infection [164].

4.3.2. CXCL 10

CXCL 10 is a molecule with various biological functions and is a member of the interferon-inducible tripeptide motif Glu-Leu-Arg-negative (ELR^-) CXC chemokines [165]. This chemokine, along with CXCL 9 and CXCL 11, is expressed on activated T lymphocytes and NK cells and recruits these cells to the sites of infection and inflammation via the G protein coupled receptor CXCR 3 signaling [166,167]. In addition to leukocyte recruitment, CXCR 3 ligands also show antibacterial activities, similar to cationic AMPs (including defensins) [165]. CXCL 10 promotes the chemotactic activity of CXCR 3^+ cells, induces apoptosis, regulates cell growth and proliferation, and inhibits angiogenesis in infectious and inflammatory diseases and cancers [168–171]. In previous studies, it was found that CXCL 10 was involved in the innate defense of the cornea against microbial infections. The induced and sustained expression in the inflammatory cornea may be one of endogenous factors that prevents angiogenesis and controls vascular regression [172,173]. CXCL10-CXCR3-mediated signaling pathways, play an important role in the recruitment and activation of NK cells, which can enhance innate immunity to eliminate invasive pathogens and prevent disease [174].

4.3.3. Chitinase 3-like 1 (Chi3L1)

Chi3L1 is a member of the 18 glycosyl hydrolase family in mammals. In the strong innate defense of cornea induced by flagellin (the only ligand of TLR5), the up-regulation of Chi3L1 was the most significant [175]. Considered as a key regulator of anti-infective and adaptive Th2 responses [176,177], Chi3L1 promotes bacterial clearance and enhances host defense. In the cases of fungal infections, Chi3L1 stimulates the expression of AMPs and inflammatory factors in the cornea infected by *C. albicans*, promotes the innate immunity of cornea to prevent and to fight fungal keratitis caused by *C. albicans* [174,178].

4.3.4. Clavanin A

Clavanins are antibacterial peptides expressed in the hemocytes of marine tunicate *Styela clava* [179], and they are rich in histidine, phenylalanine and glycine [180]. Among these clavanins, clavanin A can cause damages on cell membrane, leading to the death of fungal cells. In common ocular fungal pathogens, such as *C. albicans*, *A. fumigatus*, *Alternaria*, and *Fusarium*, both clavanin A and self-assembled clavanin A showed antifungal activities. In ophthalmic surgery, the rapid reconstruction of ocular surface often requires amniotic membrane (AM), on which fungal biofilms may form and serve as a reservoir for persisting infections on ocular surfaces. Coating with clavanin A can significantly reduce the fungal biofilm formation on AM, compared with uncoated AM. The underlying mechanism may be the cell membrane damages caused by the rapid dissipation of transmembrane potential [181]. Therefore, coating with clavanin A (on AM) can prevent fungal infections on the ocular surfaces during ophthalmic surgery.

There are other AMPs in the treatment of fungal keratitis. For example, *Pc*-C and *Pc*-E can reduce the binding of *A. fumigatus* to corneal epithelial cells *in vitro*, while CAP37 can kill fungal pathogens and inhibit infections [151,182].

4.4. Others

The C-type lectins are the most common PRRs during fungal keratitis immune responses, besides the TLR family [183]. Mincle expressed on the surface of macrophages, can recognizes the geometric conformation of fungal-specific α-mannosyl residues [184,185]. Research has shown it confers resistance to fungal infections and prevention from fungal diseases [185]. Mincle expression is up-regulated in early stages of fungal keratitis caused by *A. fumigatus* in rats and human [186]. Changes in inflammatory cytokines, such as TNF-α, IL-1β, IL-10 and CCL3, can affect the occurrence and development of fungal keratitis. TNF-α recruits the leukocytes and enhances its antifungal activity

[187,188]. IL-1β may be associated with the severity of diseases [189]. IL-10 inhibits the proliferation of Th1 cells, promotes the activation of Th2 cells, and inhibits the phagocytosis of fungal cells by macrophages, thus impairing the defense of host to fungal pathogens [190]. CCL3 is a key regulator of polymorphonuclear neutrophil (PMN) recruitment [191]. Therefore, Mincle can be used as a potential target for the prevention and treatment of fungal keratitis.

As a ubiquitin-like modifier with antibacterial and antiviral activities, IFN-stimulated gene of 15 kDa (ISG15) is expressed in many cells, including innate immune cells and epithelial cells [192]. Individuals with ISG15 deficiency were reportedly more susceptible to mycobacteria [193,194]. However, the role of ISG15 in fungal infections remains largely to be explored. ISG15 can induce IL-1 Ra, CXCL10 and CRAMP in mouse corneal epithelial cells, which are important media and/or effectors of innate immunity against corneal microbial infections [174,195]. In the cornea with defective ISG15 production, the fungal load, the infiltration of PMN and the expression of CXCL2 protein were significantly increased, indicating a role of ISG15 in the corneal immunity against fungi [195]. Subconjunctival injection of exogenous ISG15 in early stages of infection, can potentiate the innate immunity against *C. albicans* in corneal infections, thus reducing the severity of keratitis [195].

Contact lenses are one of the most common risk factors for fungal keratitis. Disinfection of contact lenses, the cleanness sustaining of storage solutions and cleaning solutions can prevent fungal keratitis associated with contact lenses. A new povidone-iodine (PI) disinfection system can kill all bacteria, Acanthamoeba and fungi, after a 4-h period of disinfection and cleaning, helping prevent microbial keratitis associated with contact lenses [196]. In addition, the bacteriostatic 0.01% hypochlorous acid has rapid fungicidal activity against ocular fungal pathogens, including common yeasts and molds [197]. Therefore, it can be applied to the disinfection of contact lenses. Recently, an econazole-eluting contact lens has been introduced, which can be loaded with antifungal drugs. The sustained release of the drug can last for three weeks, holding long-term antifungal effects on *C. albicans*. This regime helps to improve patient compliance and may represent a new method to prevent or treat fungal diseases [198].

5. Conclusions

More than 70 fungal species can cause sight-threatening keratitis, the pathogenesis of which involves morphological changes, adhesion, ocular trauma, virulence factors and immune evasion. Fugal keratitis is difficult to treat, due to the scarcity of antifungal drugs. The emergence of drug-resistant fungal strains and the lack of cornea donors necessitate the early diagnosis and prevention of fungal keratitis. In contrast to traditional detecting methods, new methods based on molecular biology and imaging, such as PCR, DNA sequencing and IVCM, can improve the diagnosis and optimize the treatment to obtain a better prognosis. Developing vaccines and novel antifungal molecules may contribute to the prevention of fungal keratitis, however, there is no real clinical prophylaxis. As far as individuals are concerned, keeping immune homeostasis and good hygiene habits is critical for the prevention. With the progresses in pathogenesis and diagnosis of fungal keratitis, more insightful prevention strategies, as well as treatments, undoubtedly will be developed to reduce the socioeconomic burden imposed by fungal keratitis.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2019.103802.

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