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A novel murine model of *Fusarium solani* keratitis utilizing fluorescent labeled fungi

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ABSTRACT

Fungal keratitis is a common disease that causes blindness. An effective animal model for fungal keratitis is essential for advancing research on this disease. Our objective is to develop a novel mouse model of *Fusarium solani* keratitis through the inoculation of fluorescent-labeled fungi into the cornea to facilitate the accurate and early identification and screening of fungal infections. *F. solani* was used as the model fungus in this study. In in vitro experiment, the effects of Calcofluor White (CFW) staining concentration and duration on the fluorescence intensity of *F. solani* were determined through the mean fluorescence intensity (MFI); the effects of CFW staining on the growth of *F. solani* were determined by the colony diameter. In in vivo experiment, the *F. solani* keratitis mice were induced and divided into a CFW-unlabeled and CFW-labeled groups. The positive rate, corneal lesion score and several positive rate determination methods were measured. The MFIs of *F. solani* in the 30 μg/ml CFW-30 min, 90 μg/ml CFW-10 min and 90 μg/ml CFW-30 min groups were higher than that in the 10 μg/ml CFW-10 min group ($P < 0.01$). Compared with the 30 μg/ml CFW-30 min group, only the 90 μg/ml CFW-30 min group showed higher MFI ($P < 0.05$). No significant difference was observed in the colony diameter in the CFW unstained group compared with that in the 10, 30, 90, 270, or 810 μg/ml CFW groups stained for either 10 or 30 min ($P > 0.05$). No significant differences ($P > 0.05$) were observed for the positive rate or the corneal lesion scores between the CFW-unlabeled and the CFW-labeled group. On day 1 and 2, the positive rates of the infected corneas in the scraping group were lower than those in the fluorescence microscopy group ($P < 0.05$). On day 3, these observe methods showed no significant difference ($P > 0.05$). Thus, these experiments established a novel murine model of *F. solani* keratitis utilizing fluorescent labeled fungi. This model facilitates the accurate identification and screening of fungal infections during the early stages of fungal keratitis and provides a novel and reliable technology to study the fungal keratitis.

1. Introduction

Fungal keratitis is a common disease that causes blindness. The incidence of fungal keratitis is primarily observed in China, India and other developing countries (Wang et al., 2009, 2000; Chowdhary and Singh, 2005; Bhartiya et al., 2007). An effective animal model for fungal keratitis is essential for advancing research on this disease. However, obtaining a method to accurately identify and screen fungal infection during the early stages in keratitis models is currently a challenge for researchers in this field. Although corneal scraping is used for early diagnosis, the positive detection rate for fungal keratitis is as low as 60% in mice, reflecting differences in the cornea between humans and mice (Zhang et al., 2010). The detection of hyphae though in vivo confocal microscopy is another method used in the early diagnosis of keratitis, but the failure to find the hyphae results in false negative diagnoses. Moreover, in vivo confocal microscopy is not widely used in laboratories. Positive fungal cultures from infected corneas represent the gold standard for identifying fungal infections, but collecting specimens for microbiological culture from the lesions of the cornea is not feasible except at the end of the experiment.

The detection of fluorescent labeled fungi using fluorescence microscopy is a simple and sensitive method. Labeling fungi

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through the insertion of a fluorescent protein gene into the fungal genome is also a plausible method; however, this process is cumbersome and requires specific expertise and resources. Therefore, other simple methods for the in vivo labeling of fungi are needed.

Calcofluor White (CFW) is a non-specific fluorochrome that binds to cellulose and chitin, which are components of the cell wall of fungi, through intercalation into nascent chitin chains. CFW is a dye commonly used in the clinical laboratory for the detection of fungi in a fixed sample. Recently, CFW was used in fungal research as a vital, although there has been some debate about whether these dyes might also be internalized and transported to cells through other mechanisms. For example, fungal conidia are sufficiently labeled using CFW, although these specimens typically observed for less than 10 h (Bruns et al., 2010; Luther et al., 2006). To date, few experiments have been conducted concerning the optional concentration and duration of CFW staining for labeling live fungal hyphae. Furthermore, the potential influence of long-term CFW vital labeling on fungal growth remains undetermined.

In this paper, the effects of concentration and duration of CFW staining on the fluorescence intensity and growth of *Fusarium solani* were investigated. Using an optimal CFW staining concentration and duration, we tried to develop a novel mouse model of *F. solani* keratitis through the inoculation of fluorescent labeled fungi into the cornea to facilitate the accurate and early identification and screening of fungal infections. Using this model, fungal growth in the cornea was clearly observed using fluorescence microscopy.

2. Methods and materials

2.1. Determination of the fungal fluorescence intensity at different CFW concentrations and durations

F. solani (strain number: CGMCC 3.1791) was purchased from the China General Microbiological Culture Collection Center. *F. solani* was passaged in accordance with standard methods and used after 2–3 generations. Two milliliters of sterile saline was added to the slant medium containing well-grown *F. solani*. The fungal hyphae were ground using a sterile glass rod to prepare the fungal suspension. The suspension was centrifuged at 2000 rpm for 10 min and washed to prepare 10, 30 and 90 μg/ml CFW M2R (Sigma–Aldrich, USA) fungal suspensions. Each concentration was incubated for either 10 or 30 min, and 2 μl of each suspension was transferred to a slide using a micropipette. The specimens were scanned on a single-photon confocal laser scanning microscope (Nikon C1si, Japanese) using a 408 nm laser and 515/30 nm filters. All groups were scanned using the following parameters: a 1.9% excitation light intensity, a gain value of 90, and a pinhole of S. Ten representative fungi were randomly selected from each group for the determination of the mean fluorescence intensity (MFI) of the target fungus. The MFI value was obtained automatically obtained by drawing the determined target fungal followed the fungal boundary using Nikon AR software.

2.2. Growing assays for CFW-labeled F. solani

Potato Dextrose Agar (PDA) plates were inoculated with well-grown fungi and incubated at 28 °C in a constant-temperature incubator for 3–5 days. Blank PDA plates were punched with holes in the center using a 2-mm-diameter puncher according to a previously described method (Butty et al., 1995; Shiburaj, 2011). Additional well-grown colony plates were also punched with holes at locations one-third of the distance from the fringe to the center of the colony. The agar plugs were carefully transferred to small plates, immersed in 10, 30, 90, 270 or 810 μg/ml CFW solution, and incubated in the dark for 10 or 30 min. The labeled agar plugs were inoculated into the center hole of the blank PDA plates, and cultured at 28 °C in the dark in a constant-temperature incubator. The colony diameter was measured once a day until the colonies covered the entire plate. The growth curve of the fluorescent-labeled *F. solani* was drawn based on growth time and the diameter of the colony.

2.3. Preparation of a mouse model of F. solani keratitis

C57BL/6J mice were acquired from the Model Animal Research Center of Nanjing University (Nanjing, China). All mice were used at 10–14 weeks of age. The cares and experimentation protocols for the animal work were approved through the Ethical Committee of Experimental Animal Care of Henan Eye Institute in compliance with the National Institutes of Health guidelines. All procedures used in this study were performed in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research of the Association for Research in Vision and Ophthalmology.

The mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (80 mg/kg.b.w.) (Sigma–Aldrich, USA). The corneal surface was anesthetized using 1% tetracaine hydrochloride eye drops. A dissecting microscope (Topcon OMS-90, Japanese) and a 2-mm trephine were used to mark a circular area in the central cornea. A sterile scalpel blade (carbon steel, size 11, Shanghai Pudong Jinhuan Medical Products Co. Ltd., China) was used to create a cross scratch within this circular area at a depth exceeding the Bowman's membrane. A sharpened bamboo toothpick (0.30 mm tip diameter, 1.10 mm tip length) was used to scrape along the scratch 2–3 times to create a rough surface. The scratched cornea was subsequently smeared with fungi, which were unstained or stained with CFW; using the tip of a sharpened bamboo stick. The mouse model of *F. solani* keratitis was developed according to a previously described method (Zhang et al., 2012). The mice were divided into two groups: the *F. solani* CFW-unlabeled and CFW-labeled group. The labeled fungi were stained using 30 μg/ml CFW for 30 min. The mice were housed in the dark to avoid fluorescence quenching.

2.4. Measurement of the positive rate for the development of F. solani keratitis

Twenty-four hours after inoculation, infected cornea tissue samples were collected from 30 corneas from the CFW-unlabeled and CFW-labeled groups. These samples were transferred to PDA slant media, incubated in a 28 °C incubator with daily observation. A positive model was defined as the growth of *F. solani* in these cultures within two weeks.

2.5. Corneal lesion scores

The severity of keratitis in the animals was scored daily using a dissecting microscope and a slit lamp from day 1–10. A grade of 0–4 was assigned to each of the following criteria (Wu et al., 2003; Ozturk et al., 2007): area of opacity, density of opacity, surface regularity and other variables. The grades for the area of opacity were as follows: grade 1 (1–25% of total corneal area), grade 2 (26–50% of total corneal area), grade 3 (51–75% of total corneal area), and grade 4 (76–100% of total corneal area). The grades for the density of opacity were as follows: grade 1 (slightly misty opacity of cornea, relatively clear and pupil iris), grade 2 (opacity of corneal superficial layer, visible pupil and iris through the lesion), grade 3 (uneven opacity of whole corneal layer), and grade 4 (even and