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Original Article

Detection of cutaneous protothecosis by fluorescence *in situ* hybridization

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Abstract: *Objective:* To evaluate the value of fluorescence *in situ* hybridization (FISH) as a tool for the detection of cutaneous protothecosis. *Methods:* A mouse model of cutaneous protothecosis was established by subcutaneous injection of *Prototheca zopfii*. Control mice were injected with *Candida albicans*, *Cryptococcus neoformans* or PBS. Skin samples were harvested 7 days after injection and embedded in paraffin for staining and labeling, or put into culture for detection of microorganisms. Skin sections were stained with Periodic acid-Schiff (PAS) and hematoxylin-eosin (HE). A *Prototheca*-specific DNA probe was developed and tested on the cultures of *Prototheca zopfii*, *Candida albicans*, *Cryptococcus neoformans*, then used for FISH on skin sections from *Prototheca*-infected and control mice. *Results:* All of the 11 mice injected with *Prototheca* developed cutaneous lesions that were positive for *Prototheca* upon culturing. The *Prototheca* FISH probe reacted strongly with cultured *Prototheca,* but failed to react with cultured *Candida albicans*or *Cryptococcus neoformans*. Skin sections from *Prototheca*-infected mice gave a positive signal with the *Prototheca* probe, while skin sections from control mice did not. *Conclusions: Prototheca* in paraffin-embedded skin tissue sections can be rapidly and accurately identified by FISH. This new method could be useful in the diagnosis of cutaneous protothecosis in the clinic and in epidemiological studies.

Keywords: Fluorescence *in situ* hybridization, oligonucleotide probe, *prototheca*, skin, paraffin section

Introduction

Prototheca is an opportunistic pathogenic microalga ubiquitous in nature. *Prototheca* mainly exists in the environment, including soil, fresh and salt water, tree bark, sewage, animal waste and some types of food [1]. The disease caused by prototheca includes three pattens: cutaneous, olecranon bursitis and systemic. The cutaneous form is the most common, and mainly caused by *P. wickerhamii* and *P. zopfii* in immunocompromised patients [2, 3]. Cutaneous protothecosis has various clinical manifestations, including erythema, papules, nodules and plaques, as well as superficial ulcers, verrucous hyperplasia, herpes-like lesions, lichenification, purulent discharge and atrophy [4]. Most of the cutaneous protothecosis occur on the exposed areas, such as face and extremities, and is closely related to multi-pathogen invasion after traumatic damage [5]. Infections can occur in both immunocompetent and immunosuppressed patients, but severe and disseminated infections tend to occur in immunocompromised individuals [6].

Only 16 cases of protothecosis (10 cutaneous) have been reported in the Chinese mainland [7-9], while only 7 cases (all cutaneous) have been reported in Chinese Taiwan and Hong Kong. The higher incidence in mainland China may be due to the environmental factors, higher use of agents such as glucocorticoids and chemotherapeutics which reduce immune responses, and more awareness of protothecosis. The actual cutaneous protothecosis infection rate in China may be even higher than the reported rate [10].

Prototheca are sometimes noted on routine Periodic acid-Schiff (PAS), Hematoxylin and Eosin (HE) and Gomori methenamine-silver histochemical staining [4], but since some fungi have similar morphology to *Prototheca*, simple histopathology is not an effective method for identifying *Prototheca*. Sabouraud dextrose

agar culture allows definitive identification [4]; however, some samples fail to be cultured due to certain unfavorable conditions *in vitro*. Recently, molecular biology technology has been used to detect *Prototheca*, including two-step Real Time PCR reaction followed by DNA Resolution Melting Analysis (qPCR/RMA) [19] and PCR genotyping and mass spectrometry proteomic phenotyping [18]. These methods are time consuming and can have highly variable results.

Fluorescence *in situ* hybridization (FISH) is a powerful technique that uses non-toxic fluorescent DNA probes to target specific sequences within a nucleus, resulting in signals that can be detected under a fluorescence microscope. It is frequently used to detect bacterial species in environmental and clinical samples and species-specific probes have been developed for the rapid identification of pathogenic species [20, 21]. A number of studies showed that FISH could accurately identify fungal organisms, including clinically relevant yeasts such as *Candida, Cryptococcus* species, *aspergillus*, *penicillium mameffei,* and *fusarium* [22-25]. However, specific probes for the diagnosis of cutaneous protothecosis have not been available. In the present study, we developed and tested a specific FISH probe for *Prototheca*.

Materials and methods

Prototheca and fungal strains and culture conditions

The strain of *Prototheca* used in this study was provided by the Center for Medical Mycology, Huashan Hospital, Fudan University, Shanghai, China. The strain was isolated from a patient who suffered from cutaneous protothecosis. It was identified as *P. zopfii* by morphology and molecular biology [11] and was preserved in the American Type Culture Collection (ATCC, MYA-4789). *Prototheca* was inoculated in Sabouraud liquid medium (Sigma-Aldrich, USA), and cultured at 28°C for 7 days before further use.

Candida albicans and *Cryptococcus neoformans* were obtained from American Tissue Culture Collection (ATCC) and were cultured in Sabouraud and YPD medium, respectively.

Mouse model of cutaneous protothecosis

A total of 20 male BALB/c mice aged 6-8 weeks and weighing 18-22 g were provided by the Animal Research Center of Fudan University. All mice were housed in a local facility for laboratory animal care and fed *ad libitum* on stock diet. This study was approved by the Ethics Committee for Animal Care and Use, Fudan University, according to generally accepted international standards.

The mouse model of cutaneous protothecosis was established according to a previously published method [12]. Briefly, *Prototheca* culture was diluted to 1×10^9 CFU/ml with PBS, and 200 μl was injected subcutaneously in the abdomen of each mouse according to a preliminary study (n=11). For negative controls, mouse was injected with 0.5 ml 2×10^7 CFU/ml *Candida albicans* through the tail vein (n=3), or with 1.0 ml 2×10^7 CFU/ml *Cryptococcus neoformans* intraperitoneally (n=3), or with the same volume of PBS (n=3). After 7 days mice were sacrificed by cervical dislocation and abdominal skin samples were isolated sterilely. For each mouse injected with *Prototheca*, 3 samples of skin lesions were collected: one for culture, one for direct microscopic examination, and one for pathology. For control mice, skin samples were taken for microscopic examination and for pathology. For pathology, the skin samples were fixed with 10% formaldehyde solution for 24 hours and embedded in paraffin, and three serial 4 μm sections were cut for each sample. One section was used for FISH (see below) and the other two were used for PAS and HE staining, which were performed in the Department of Pathology at Huashan Hospital, Fudan University, by the physicians who have working experience over ten years, according to standard methods [13].

FISH probe for detection of prototheca

The DNA probe for FISH was designed using Genius software, optimized manually, and its specificity was validated by BLAST according to published methods [14, 15]. The FITC-labeled probe with the sequence 5'-CGACGACTC TCCCAACCCGCG-3' was synthesized by Invitrogen Inc. The probe was stored at -20°C and protected from light. The specificity and effectiveness of the probe were validated by labeling cultured *Prototheca*, *Candida albicans* and *Cryptococcus neoformans.*

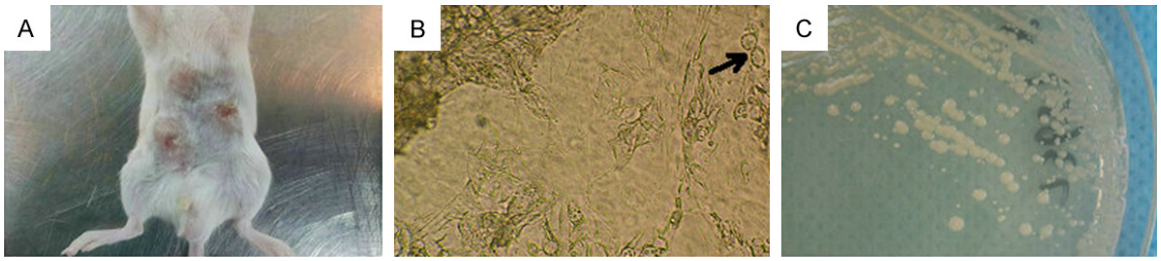

Figure 1. Analysis of mouse skin lesions 7 days after infection with *Prototheca*. A: Abdominal skin. B: Microscopic analysis of skin lesion sample. Black arrow indicates spores (Magnification=400×). C: Culture of skin lesion sample.

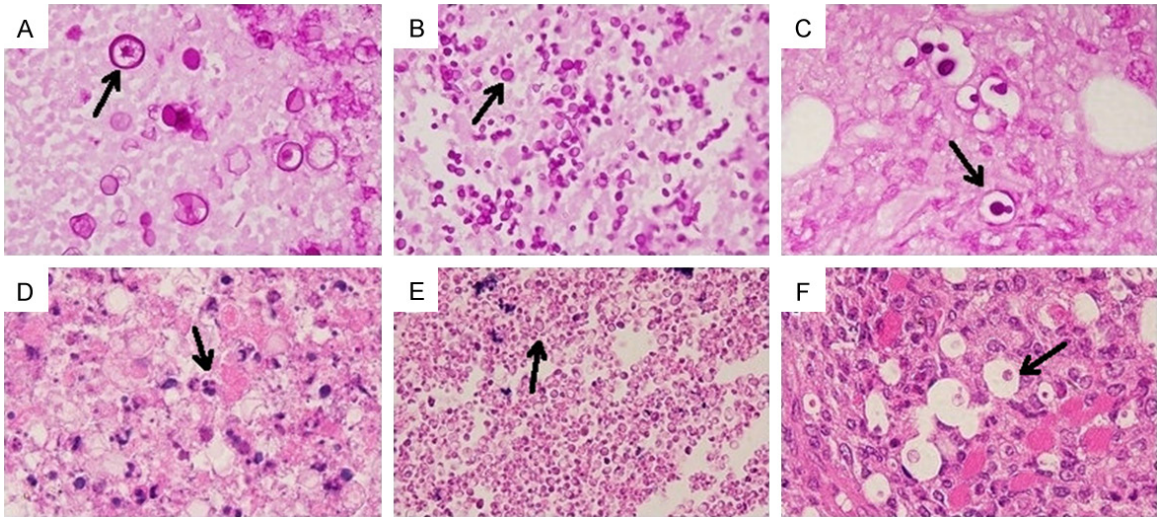

Figure 2. Histological staining of cutaneous tissue from *Prototheca*-infected and control mice. A-C: PAS staining; D-F: H&E staining. A, D: Sections from *Prototheca*-infected mice; B, E: Sections from *Candida albicans*-infected mice; C, F: Sections from *Cryptococcus neoformans*-infected mice. Black arrows indicate algae or fungal elements. Magnification is 400×.

Fluorescence in situ hybridization (FISH)

The FISH assay was performed as described previously [16, 17]. Briefly, paraffin embedded tissue sections were deparaffinized in 100% xylene for 5 minutes, followed by 5 min incubations in decreasing concentrations of ethanol from 100% to 75% (in distilled water). The sections were incubated with a cetyltrimethylammonium bromide (CTAB) solution (0.8 mol/L NaCl, 50 mmol/L EDTA, 0.1% SDS and 1% CTAB) in a 60°C water bath for 20 min. The sections were heated at 80°C for 6 minutes, transferred to cold ethanol of increasing concentration (70%, 80% and 100%), 2 minutes at each concentration, and then air-dried. For hybridization, 10 μL of 1 μmol/L probe solution was dropped on the sections and covered with cover glass, sealed with chrome alum gelatin, and incubated at 37°C in the dark for 6 hours. After hybridization, the sections were washed twice with 2×SSC (750 mmol/L NaCl, 75 mmol/L sodium citrate, pH 7.0) at room temperature for 3 minutes. The sections were then dehydrated gradually with ethanol (70%, 80% and 100%, 2 minutes at each concentration), and were air-dried. Finally, the sections were covered with cover glass and observed with Olympus X51 (Japan) microscope for fluorescence determination. Images were taken with an Olympus 1X51 camera and processed with Photoshop CS9.0 software.

Results

Mice inoculated with *Prototheca* showed signs of established cutaneous protothecosis seven days after inoculation, including papules, nodular abscesses, ulcers and scabs at the inoculation site (**Figure 1A**). The infection rate was

Figure 3. Verification of *Prototheca* FISH probe. A: Cultured *Prototheca* labeled with probe. B: Cultured *Candida albicans* labeled with probe. C: Cultured *Cryptococcus neoformans* labeled with probe. Magnification is 400×.

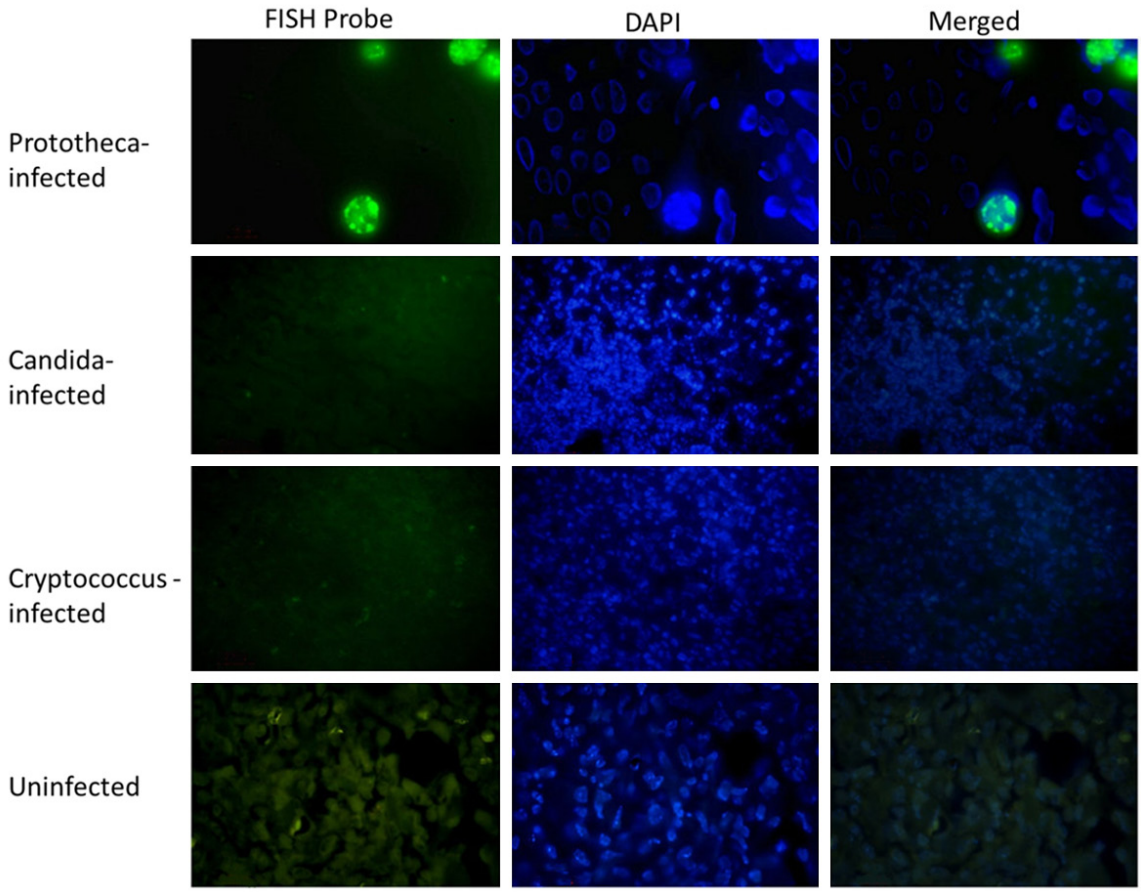

Figure 4. FISH on cutaneous tissue from *Prototheca*-infected and control mice using the *Prototheca* probe. Sections of paraffin-embedded cutaneous tissue from *Prototheca*-infected and control mice were labeled with *Prototheca* probe (green) and DAPI (blue) as described in the Methods section. Magnification for *Prototheca* infected is 1000×. Magnification for others is 200×.

100%. Direct microscopic examination of skin samples from inoculated mice showed that the lesions contained several colorless and transparent endogenous sporangia of various sizes (**Figure 1B**). When skin from *Prototheca* inoculated mice was cultured in Sabouraud medium, cream-colored yeast-like colonies with smooth surfaces grew out (**Figure 1C**).

PAS and HE staining of skin from *Prototheca*-inoculated mice showed small clusters of non-budding round to oval microorganisms resembling fungal elements in the dermis. The sporangia were round to oval and had regular internal divisions, presenting the typical appearance of *Prototheca* (**Figure 2A** and **2D**). PAS and HE staining for *Candida albicans* (**Figure**

2B and **2E**) and *Cryptococcus neoformans* (**Figure 2C** and **2E**) were shown for comparison.

A FISH probe for *Prototheca* was developed and its effectiveness and specificity were initially tested by hybridizing to cultured *Prototheca, Candida albicans* and *Cryptococcus neoformans* cells (**Figure 3**). The probe labeled *Prototheca* cells very well, and did not react with the yeast strains tested.

The FISH probe was then hybridized to skin sections from infected and uninfected mice. Before hybridization skin sections were incubated with CTAB, which has been shown to increase the penetration of DNA probes into *Prototheca* [16] and to increase hydrophobic interactions and/or electrostatic attraction between probe and target [26]. Skin sections from all of the 11 *Prototheca* infected mice gave a positive FISH reaction with the *Prototheca* probe, with granular, focal or scattered appearance. No FISH reaction was observed in skin sections from uninfected mice (**Figure 4**).

Discussion

In this study, we showed that the probe we developed can specifically identify *Prototheca* in skin samples from infected mice via the FISH procedure, opening up the potential use of FISH in the diagnosis of cutaneous protothecosis.

Because PCR technology is simple and convenient, it has been used in many fields, including the identification of fungi. But its result is affected by many factors, such as amplification reaction inhibitor during the purification of the specimen or nucleic acid, the differences of temperature between the instrument hole, and random error in the nucleic acid extraction, all these could lead to negative results. Therefore, the clinical application of PCR must have strict laboratory division, laboratory management and quality control measures to ensure the accuracy of the results.

Compared with traditional PCR methods of identifying *Prototheca*, FISH has following advantages: it only requires a few hours for the assessment; it is unnecessary to extract DNA; *Prototheca* is preserved well in the paraffin embedded tissue; the amount of the biopsy material required for FISH is very small.

There are also some disadvantages using FISH as a diagnostic tool. This technique requires fluorescence microscopy and an imaging analysis system, which are not available in many laboratories. It also requires careful optimization and appropriate controls. Background fluorescence from cells or environmental sample material can interfere and penetration of the probe can be inhibited by the structure of the cell wall. In bacteria the amount of rRNA may be too small to allow detection by FISH.

In conclusion, we have shown that FISH on sections of paraffin-embedded cutaneous tissue can be an effective and specific tool for identification of *Prototheca* infection. This method will provide a new approach for quick and accurate diagnosis of cutaneous protothecosis, and could be used in epidemiologic studies of protothecosis as well.

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Disclosure of conflict of interest

None.

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