

Preliminary Study on Solid-phase Hybridization for Detection of Common Pathogenic Bacteria Causing Fungal Keratitis

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Abstract: Objective: To develop an experimental method for the rapid detection and accurate identification of common pathogenic bacteria causing fungal keratitis in China based on the gene chip principle and by the solid-phase hybridization technology. **Methods:** For the 6 categories and 12 species of common clinical fungi causing keratitis in China including *fusarium solani*, *fusarium moniliforme*, *fusarium poae*, *fusarium oxysporum*, *aspergillus fumigatus*, *aspergillus flavus*, *aspergillus terreus*, *aspergillus niger*, *curvularia lunatus*, *penicillium implicatum*, *alternaria alternate*, and *candida albicans*, fixed a specific oligonucleotide probe onto an aldehyde slide. Amplified the above fungi with a pair of fluorescence labeling universal primers, hybridized the fluorescence labeling amplified product with the probe arrayed in the slide and observed the color under a fluorescence microscope. **Result:** Through observation of agarose gel electrophoresis, 12 species of fungi all produced about 530-630 base pair PCR amplification products; conducted hybridization detection of them under the same conditions and obtained post-hybridization fluorescent color maps with their own respective characteristics; different bacteria can be directly differentiated and judged through fluorescence signals. **Conclusion:** The 12 common clinical species of pathogenic bacteria causing fungal keratitis in our country can be detected within 3-4 hours with fluorescence labeling universal primers and through PCR amplification to produce fluorescence labeling amplified products and hybridize with the oligonucleotide probe on the aldehyde slide.

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Key Words: Solid-phase hybridization, Fungal keratitis, Pathogenic bacteria, Bacteria detection

Introduction

Fungal Keratitis is a serious cause of blindness. It is an important prerequisite to rapidly, sensitively and efficiently detect the pathogenic bacteria for prevention and cure of this disease and effective reduction of the blindness rate. We attempt to rapidly detect the common pathogenic bacteria causing fungal keratitis with solid-phase hybridization technology.

1. Materials and Methods

1.1 Materials (1) Standard strains *Fusarium solani* (3.1792), *fusarium moniliforme* (3.752), *fusarium poae* (3.4601), *fusarium oxysporum* (3.4743), *aspergillus fumigatus* (3.722), *aspergillus flavus* (3.2758), *aspergillus niger* (3.759), *aspergillus terreus* (3.3935), *penicillium implicatum* (3.512), *curvularia lunatus* (3.1471), *alternaria alternate* (3.577) and *candida albicans* (2.538) were all bought from China General Microbiological Culture Collection Center, Institute of Microbiology, Chinese Academy of Sciences. (2) Main Instruments PTC-200 Gene Amplifier (US MJ Company), PCA300 Electrophoresis Apparatus (BIORAD Company), Centrifuge 5402 (Eppendorf Company), Alphaimager TM2200 Gel Imaging System (US Alpha Company) and BX51 Fluorescence Microscope (Japanese Olympus Company). (3) Main Reagents Reagents used in DNA extraction, polymerase chain reaction

and hybridization were provided by Sino-American Biotechnology Co., Ltd.; slide treatment agents, 3-aminopropyl triethoxysilane and sodium borohydride were bought from Sigma Company; glutaraldehyde was bought from Sangon Biotech (Shanghai) Co., Ltd. **1.2 Methods** 1) Design of PCR primer and oligonucleotide probe. Obtained the gene sequence of 10 species of fungi from the Genbank, conducted sequence alignment for the retrieved fungal genomic DNA and obtained the base alignment chart and cluster analysis result. Looked for the primers in the conservative range with the Primers program. Analysed ITS1 and ITS2 specific variable region, conducted BLAST alignment and selection and designed the specific oligonucleotide probes for the 10 species of fungi with the Oligo software. The primers and probes were both synthesized by Sangon Biotech (Shanghai) Co., Ltd (Table 1). (2) Fungal DNA extraction Took 3ul as the reaction template with the guanidine isothiocyanate method [1]. (3) PCR amplification reaction system. The total reaction volume is 30ul, the 20× reaction buffer is 1.5μl, MgCl₂ solution is 2.0mM, dNTP mixture is 200μM, primers are 6pmol×2, Taq DNA polymerase is 2u and the template DNA is 3ul. The reaction condition is 94°C 5min and 1 cycle; 94°C45s, 55°C60s, 72°C 60s and a total of 36 cycles; 72°C5 min 1 cycle. (4)

Surface treatment of the slide carrier. Selected the domestic microscope slide as the carrier. Then cleaned it with distilled water and dried it by centrifugation after it was immersed in the chromic acid lotion overnight; Next, immersed it into 25% ammonia water overnight, cleaned it with distilled water and dried it by centrifugation; immersed it into 95% ethanol (PH 4.5) containing 3% 3-aminopropyl triethoxysilane, placed it into the shaker for 60 min, conducted ultrasonic cleaning with 95 % ethanol (pH 4.5) and distilled water and then dried it for 15 min at 110°C; at last, immersed the slide into 5% glutaraldehyd, placed it into the shaker for 60 min, conducted ultrasonic washing twice and dried at 110°C for 15 min ready for use. (5) Oligonucleotide probe points and treatment. Made up the probe solution with a final concentration of 50 μ mol/L, took 5 μ l from each probe solution, dissolved them into 5 μ l 3 \times SSC separately and spotted on the corresponding section of the slide with the point-like needle (Table 1). Placed the spotted slide overnight at room temperature and 80°C hydrated 2h. At first, cleaned the slide with 0.2% SDS and distilled water, then put it into NaHB4 blocking solution for 5 min and next cleaned it with 0.2% SDS and distilled water, ready for use after it was dry at room temperature. (6) Hybridization detection. Spotted hybridization solution A 50 μ l on the position of the slide fixed with a probe, took amplified product 15 μ l and mixed it evenly with hybridization solution C, which was fully mixed with solution A on the slide. Then, Made the solution take a water bath in the wet box for 1 h and then rinsed it with eluate I (1 \times SSC / 0.2 % SDS) for 1 min, eluate II (0.1 \times SSC / 0.2% SDS) for 1 min and eluate III (0.1 \times SSC) for 1 min. Then, observed its color situation under the fluorescence microscope after it dried at room temperature.

2. Results

2.1 PCR amplification result. The universal primers can separately amplify PCR amplified products of standard strains from 10 species of common clinical pathogenic fungi causing keratitis and gel electrophoretogram (Figure 1).

2.2 Gene chip hybridization result Hybridized the PCR amplification products of 10 species of fungi strains separately with the slide spotted with 10 kinds of specific probes and one kind of universal probe and there appeared fluorescence in the positions that were fixed with corresponding probes. The result showed that the 10 kinds of probes all have high specificity and each kind of probes only react with its own corresponding PCR amplified products (Figure 2).

2.3 Sequencing result of amplified products After sequencing the PCR products of 10 species of

common clinical fungi strains causing keratitis, compared the sequencing result with the nucleic acid amplification sequence selected by this test. The result showed they have exactly the same sequence, which verifies the credibility of the hybridization result.

3. Discussion

Fungal Keratitis is a serious cause of blindness and its incidence is rising rapidly in our country. Rapid and accurate detection of pathogenic bacteria can provide timely guidance for the clinical diagnosis and treatment and effectively reduce the rate of blindness. Currently, the traditional laboratory diagnostic methods are mainly microscopic examination of corneal scraping and fungal culture. They require cumbersome and time-consuming technology and are not conducive to clinical treatment guidance^[2]. Gene chip is a molecular biology technology that newly emerged in the late 20th century. It fixes cDNA and the oligonucleotide probe onto the film base surface to form a micro-array. Because the specific position of the probe in the carrier's micro-array is preset, just by the judgment of the probe signal, the change of the gene expression and structure can be inferred. Its biggest advantage is its high-throughput, miniaturization and automated analysis of the genetic information^[4]. But it also has some problems. For example, the gene chip's preparation, detection and analysis require specific equipment and software which are quite expensive and mostly used in scientific experiments and research. Therefore, it has broad prospects for the gene chip technology to be used in the detection of pathogenic microorganisms according to our national situation. We designed the universal primer and specific probe by use of the fungal ribosomal RNA genes and conducted rapid detection of the common domestic clinical 12 species of pathogenic bacteria causing fungal keratitis in accordance with the gene chip principle, with the domestic slide as the carrier and on the basis of the epidemiological survey^[5].

By use of the computer software and in combination with artificial alignment, we found the most conservative primer and the best specific oligonucleotide probe in the fungal ribosomal RNA gene sequence^[6] which is of great significance to the fungal classification within the category and among species as well as individual differences within the category. Meanwhile, we took full account of Tm, GC content, hairpin structure and other factors, tried every means to make them reach unanimity and strove to guarantee that the probes fixed onto the same carrier can obtain the best effect under the same conditions of hybridization and fluorescence excitation, thereby ensuring the success of the detection.

In accordance with the oligonucleotide

covalently fixed principle [7,8] in the gene chip preparation, we made the aldehyde slide by ourselves and used it as the carrier to conduct solid-phase hybridization and made the amino-modified oligonucleotide probe fix on the slide by the covalent way. Amplified the standard strains of 12 species of fungi with PCR technology and further amplified the corresponding gene fragments of various fungi. On the basis of the electrophoresis detection, conducted hybridization verification on the products with the specific probe fixed onto the slide, avoiding the false positive and false negative result and overcoming the deficiency that the gel electrophoresis bands of PCR products are unable to accurately differentiate the differences within the category and among the species [9,10]. The test results show that no non-specific cross-reactivity is seen on the probe fixed onto the self-made aldehyde slide and it can conduct type testing among fungi species, showing good specificity and sensitivity in the detection of standard strains. When the concentration of the PCR products were diluted until no bands were seen in the agarose gel electrophoresis, there was still fluorescent color in the chip hybridization, indicating that the sensitivity of the chip during the detection was higher than agarose gel electrophoresis after the conventional PCR. In addition, in the hybridization reaction, we used chemical methods and made the double-stranded DNA of PCR products decompose into single-stranded one, eliminating the pre-hybridization process, reducing the processing steps and shortening the hybridization time. The credibility of the hybridization results were verified by sequencing the amplified products. Thus, we could finish the strain identification of 12 species of common domestic clinical pathogenic fungi causing keratitis within 3-4 hours through PCR amplification and the solid-phase hybridization of self-made probe carrier; moreover, we also could add other pathogenic microorganisms probes in accordance with the need of the detection and conduct type diagnosis on more keratitis pathogenic microorganisms under the multiplex PCR amplification.

In the test, we took full account of the practicality of the hybridization signal detection in the clinical work. Appropriately increased the point-like amount and point-like diameter, designed a strip of universal probe as the coordinate to read signals and additionally placed the coverslip with point-like location mark grid, which guaranteed the certainty of the probe's fixed position and the stability of hybridization. Additionally, no expensive signal reading device was needed. Only the mercury lamp was used as the fluorescence microscope to excite the light source and then the detection of hybridization signals in the corresponding point-like positions could be conducted, greatly increasing the range of clinical

applications.

This study has initially established an experimental method to detect 12 species of common domestic pathogenic bacteria causing fungal keratitis on the basis of the gene chip principle and with the solid-phase hybridization technology. This method can not only conduct rapid and convenient clinical sub-type diagnosis at low cost but also fit the large-scale epidemiological investigation. Its specificity and sensitivity will be further improved and be tested in clinical applications through the optimization and perfection of the experimental conditions.

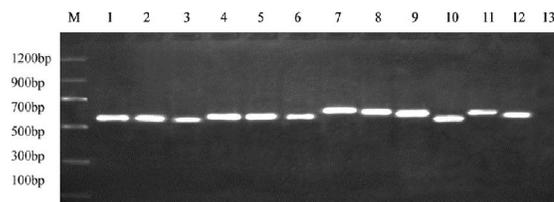


Figure 1. PCR Amplified Product Electrophoretogram

(M) DNA Molecular Weight Marker

1. *Fusarium solani*
2. *Fusarium moniliforme*
3. *Fusarium poae*
4. *Fusarium oxysporum*
5. *Aspergillus fumigatus*
6. *Aspergillus flavus*
7. *Aspergillus niger*
8. *Aspergillus terreus*
9. *Penicillium implicatum*
10. *Curvularia lunatus*
11. *Alternaria alternata*
12. *Candida albicans*
13. Negative control

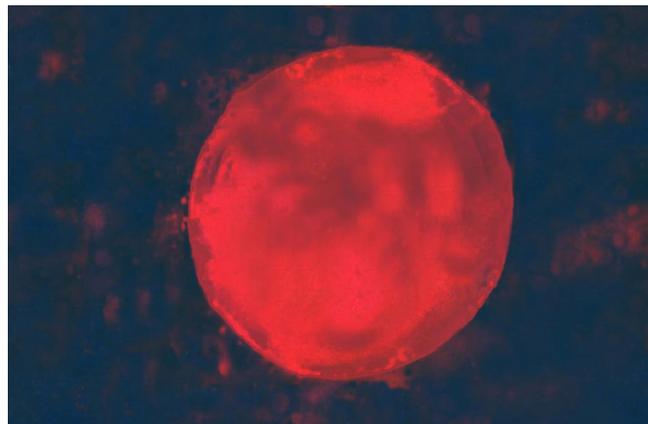


Figure 2. Positive Result of Hybridization Fluorescence Microscope 20×

Table 1. Primer & Probe

Primer & Probe	Sequence
Universal primer	
Forward	5'--tcc gta ggt gaa cct gcg g-3'
Reverse	5'-TAMRA-tcc tcc gct tat tga tat gc-3'
Probe	
Fusarium solani	5'--aga cgg ccc tgt aac aac g ata -NH ₂ -(CH ₂) ₆ -T ₁₅ -3'
Fusarium moniliforme	5'- cg agtcaaatcg cgttccc atg -NH ₂ -(CH ₂) ₆ -T ₁₅ -3'
Fusarium poae	5'- gga tca gcc cgt cct tgc tta -NH ₂ -(CH ₂) ₆ -T ₁₅ -3'
Fusarium oxysporum	5'-tag cgt agt agt aaa acc ctc gta ttc -NH ₂ -(CH ₂) ₆ -T ₁₅ -3'
Aspergillus fumigatus,	5'-cag ccg aca ccc aac ttt at cat -NH ₂ -(CH ₂) ₆ -T ₁₅ -3'
Aspergillus flavus	5'- ccg aac gca aat caa tct t aca -NH ₂ -(CH ₂) ₆ -T ₁₅ -3'
Aspergillus niger	5-ccg acg ttt tcc aac cat t caa -NH ₂ -(CH ₂) ₆ -T ₁₅ -3'
Aspergillus terreus	5'-cgc att tat ttg caa ctt gtt cat -NH ₂ -(CH ₂) ₆ -T ₁₅ -3'
Curvularia lunatus	5'-ggg tgc caa aga ctc gcc t cat -NH ₂ -(CH ₂) ₆ -T ₁₅ -3'
Alternaria alternate	5'-ata aac ctt ttg taa ttg caa tca -NH ₂ -(CH ₂) ₆ -T ₁₅ -3'
Candida albicans	5' - gg gatcgcttg acaatgg aac -NH ₂ -(CH ₂) ₆ -T ₁₅ -3'
Universal probe	5' -gca tgc atg aag aacgca gc tca -NH ₂ -(CH ₂) ₆ -T ₁₅ -3'

Table 2. Schematic Diagram of Probe Point-like Positions

1	2	3	4	5	6	13
1	2	3	4	5	6	13
7	8	9	10	11	12	13
7	8	9	10	11	12	13

1. Fusarium solani 2. Fusarium moniliforme 3. Fusarium poae
 4. Fusarium oxysporum 5. Aspergillus fumigatus 6. Aspergillus flavus
 7. Aspergillus niger 8. Aspergillus terreus 9. Penicillium implicatum
 10. Curvularia lunatus 11. Alternaria alternata 12. Candida albicans
 13. Universal probe

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