African Journal of Microbiology Research Vol. 4(19) pp. 2029-2033, 4 October, 2010
Available online http://www.academicjournals.org/ajmr
ISSN 1996-0808 ©2010 Academic Journals

Full Length Research Paper

Detection of deep fungal infection in SLE patients with multiplex fluorescent real-time PCR

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Accepted 6 August, 2010

To establish a rapid, sensitive and specific detection method for deep fungal infection, we detected the deep infection of *Candida albicans* and *Aspergillus flavus* in patients with systemic lupus erythematosus by using multiplex fluorescent real-time PCR (MFRT-PCR). Firstly, the positive rate, sensitivity and specificity of multiplex fluorescent quantitative PCR for detecting the fungal infection was tested in 20 specimens from systemic lupus erythematosus patients with *C. albicans* and *A. flavus* infections. Then, 20 specimens from SLE patients with suspected deep fungal infections and 20 other microorganism infections were detected with MFRT-PCR. Results showed the positivity rate and specificity of both 100% for detecting the deep *C. albicans* and *A. flavus* infection. Its detecting sensitivity for deep fungal infection was 75%, which was significantly higher than that of fugal culture method (40%) ($P < 0.05$). MFRT-PCR is a better method for detecting deep *C. albicans* and *A. flavus* infections, with higher sensitivity and specificity than fungal culture.

Key words: Systemic lupus erythematosus, deep fungal infection, multiplex fluorescent quantitative PCR.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a complicated disease and the patients are susceptive to infection. Deep fungus infection is one of the important reasons affecting the prognosis. Sun et al. (2003) studied the fungus infection in 427 SLE patients and they found 36 of 427 cases had fungal infection, with an infection rate of 8.43%, including 26 cases of *Candida albicans*, 4 cases of *Candida tropicalis*, 2 cases of *Candida krusei*, 2 cases of *Aspergillus*, 1 case of *Candida glabrata* and 1 case of thread fungus. SLE patients with deep fungal infection often experience a typical clinical symptoms. Moreover, the fungal culture commonly requires 2 weeks even much longer time. Therefore, the early diagnosis of SLE accompanied with fungal infection is difficult.

Multiplex fluorescent real-time PCR (MFRT-PCR) is a newly developed diagnostic technique in recent years (Fumiere et al., 2010). It can monitor several microbial species with one sample and multiple probes and obtain results within a few minutes (Bon et al., 2000; Kang et al., 2010; Zott et al., 2010; Kato et al., 2000). Moreover, it can quantitatively monitor the infected degree, which is very beneficial to the decision for illness. The application of MFRT-PCR in SLE accompanied with the deep fungus infection has not been reported. In the present study, we examined the infection of *C. albicans* and *Aspergillus flavus* in SLE patients with MFRT-PCR in order to establish a rapid, sensitive and specific monitoring method for deep fungus infection accompanied with SLE.

MATERIALS AND METHODS

Subjects

20 SLE patients with deep *C. albicans* (10 cases) and *A. flavus* (10 cases) infections in our hospital from November, 2006 to December, 2009 were enrolled in our study. MFRT-PCR was performed to detect the positive rate of infections.

MFRT-PCR and fungal culture method were used to detect the fungal infection of 20 SLE patients with the suspected deep fungal infection in order to undersand the sensitivity of these two methods. MFRT-PCR was used to detect the infection of *C. krusei*, *C. glabrata, C. tropicalis* and *A. fumigatus* of 20 other fungal micro-organism samples in order to understand the specificity of this method.

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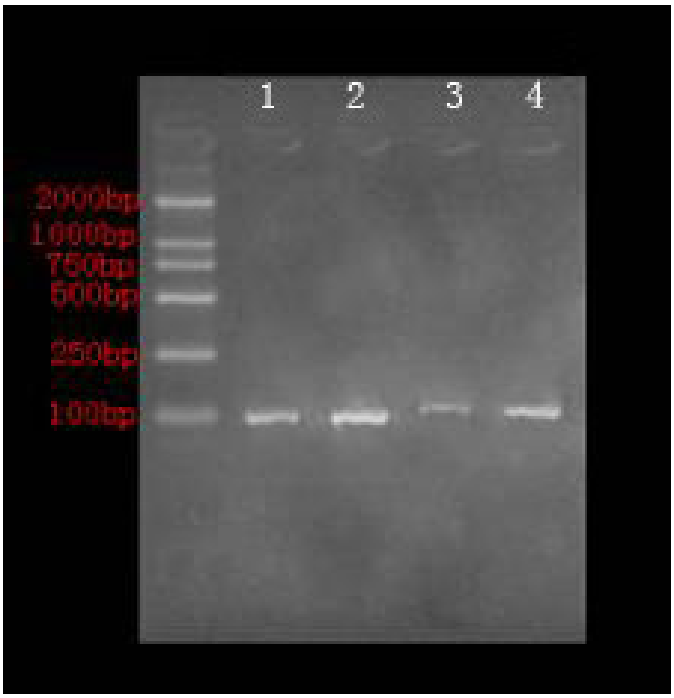

Figure 1. PCR products of *C. albicans* and *A. flavus*. Band 1 and 2 are *C. albicans* (104bp); band 3 and 4 are *A. flavus* (127 bp).

Main reagents and instruments

The primers and probes were purchased from invitrogen (USA). Taq enzyme, DNA extraction kit and fluorescence quantitative PCR kit were purchased from Guangzhou Dahui Bio Tech Co., LTD. dNTP was purchased from Sigma (USA). DNA gel purification kit was purchased from QIAquick. The standard *C. albicans* (10 cases) and *A. flavus* stain were purchased from Guangzhou microorganism centre. Horizontal strip electrophoresis apparatus, PCR machine, high-flux DNA synthesizer, fluorescence quantitative PCR instrument were purchased from Guangzhou Dahui Bio Tech Co., LTD.

Sampling

The blood sample was obtained from the cubital vena mediana. After ligating the conventional tourniquet and sterilization, the blood lancet was rapidly inserted to the cubital vena mediana. 2 ml blood sample was collected into a coagulation tube. The DNA extraction was carried out within 0.5 h.

DNA extraction

The samples and standard strains were inoculated into the autoclaved 1.5ml centrifuge tube. 50 µl blood samples was added into each tube and mixed thoroughly. The centrifuge tubes were placed into 100℃ water bath for 10 min, followed by the standing at room temperature for 10 min. The tubes were then centrifuged at 12000 r/min for 10 min. The supernatant was removed into another clean tube and stored at -20℃ for the next experiment.

MFRT-PCR

The DNA sequences of *C. albicans* and *A. flavus* were searched on ATCC website. The primers and the probes were designed. Two pairs of primers and probes were shown as follows: *C. albicans,* primers: forward: 5'-TCTCCCTCAAACCGCTGGG-3', reverse: 5'-GGTTAGACCTAAGCCATTGTCAAAG-3'. probe: JOE-CCGCCTTACCACTACCGTCTTTCAAGCAA-BHQ1a. *A. flavus,* primers: forward: 5'-GCTGCCCATCAAGCACGG-3', reverse: 5'-CCTACAGAGCGGGTGACAAAG-3'. probe: FAM-TGTGTGTTGGGTCGTCGTCCCCTCTC-TAMRA. The length of the amplified fragments was 104 and 127 bp, respectively. 50 µl MFRT-PCR reaction system contained 10 µl PCR buffer, 0.5 µl primers and probes, 1 µl Taq enzyme, 5 µl DNA template, 30 µl ddH_2O. The PCR reaction condition was as follows: pre-denaturation for 3 min at 93℃, denaturation for 45 s at 94℃ annealing and extension for 1 min at 55℃, with 45 cycles. According to the above reaction system and conditions, DNA was monitored with ABI7000 automatic fluorescence quantitative appearance. After the reaction was finished, the data were saved. The values of the samples were recorded in the Reporter window.

Statistical analysis

The data were analyzed with SPSS 13.0 software. Chi-square test was adopted to compare the rate of two samples. $P < 0.05$ was considered significant difference.

RESULTS

PCR products of *C. albicans* and *A. flavus*

Standard curve

MFRT-PCR was performed to detect the infection of *C. albicans* and *A. flavus*. The natural logarithm of the initial copy number was as the X-axis and the cycle threshold was as the Y-axis and two standard curves were obtained. The slope rate of the *C. albicans* standard curve was -3.32687, and the linear correlation coefficient R^2 was 0.996. The slope rate of the *A. flavus* was -3.31546, and the linear correlation coefficient R^2 was 0.994 (Figure 1).

Amplification picture of samples

Except for the double-negative amplification curve, the positive amplification curve showed the standard smooth shape, which suggested that the amplification was stable and the effect was good (Figures 2 - 4).

Positive rate, sensitivity and specificity

The positive rate of both *C. albicans* (10/10) and *A. flavus* (10/10) with MFRT-PCR was 100%. Therefore, the sensitivity of MFRT-PCR was 100%. For 20 samples with suspected deep fungal infection, 8 cases (40.00%) were detected with the culture method, while 15 cases (75.00%) were detected with MFRT-PCR. The sensitivity

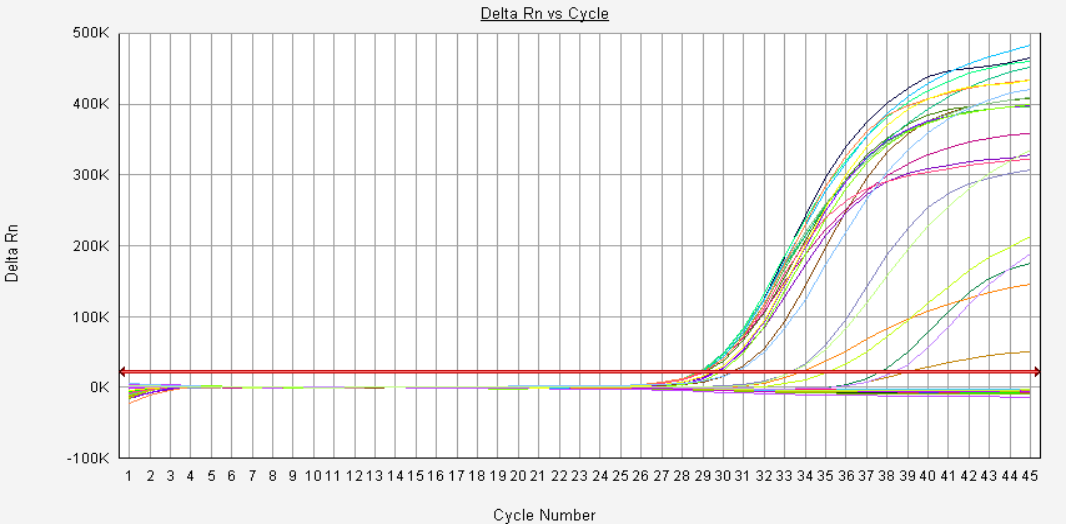

Figure 2. Amplification curve of the gene sequence of *C. albicans*. The amplification curve is standard smooth shape. The linear correlation of the initial copy number and the cycle threshold was obtained and the linear correlation coefficient R2 was 0.996.

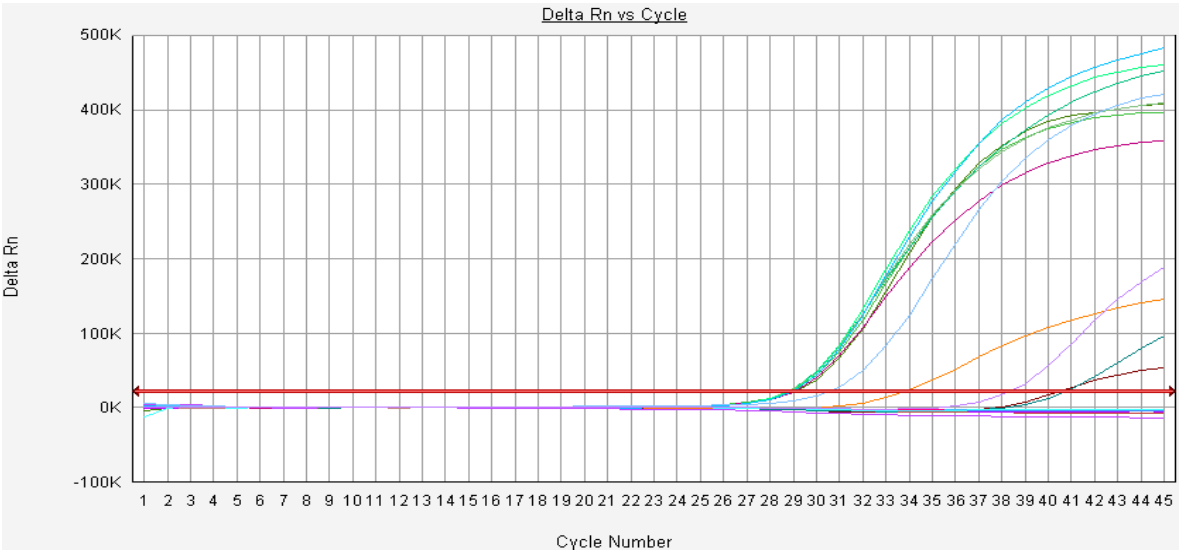

Figure 3. Amplification curve of the gene sequence of A. flavus. The amplification curve is standard smooth“ ~ ” shape. The linear correlation of the initial copy number and the cycle threshold was obtained and the linear correlation coefficient R2 was 0.994.

of these two methods was significantly different (x^2 = 5.013, P = 0.025) (Table 1). 5 *C. albicans* infection and 3 *A.* infections were detected with fungal culture, while 11 *C. albicans* infection and 4 *A.* infections were detected with MFRT-PCR. Among them, 1 case was positive for both *C. albicans* infection and *Aspergillus* infection, but the PCR copy number was low in MFRT-PCR. MFRT-PCR was used to detect the infection of 20 other fungal microorganism samples. The results showed that all the samples were negative, suggesting that the specificity was 100%.

DISCUSSION

MFRT-PCR benefited from the 5'-3' exonuclease activity of TaqDNA polymerase and the application of fluorescence resonance energy transfer (FRET) (Giulietti et al., 2001). TaqMan probe is based on the label methods of MFRT-PCR (Zhang et al., 2006; Wiwanitkit, 2010; Malmström et al., 2010). TaqMan probe which is also named hydrolysis probe is designed based on the target gene and can specifically hybridize with the amplified products. The 5’ terminal of the probe is the

Figure 4. Amplification curve of double negative samples. Neither detected the infection of *C. albicans* nor *A. flavus.*

Table 1. Sensitivity of the multiplex fluorescent real-time PCR and fungal culture method for fungal infection detection.

Groups	Negative	Positive	Total	Sensitivity (%)	x^2	P
Multiplex fluorescent Quantitative PCR	5	15	20	75.00	5.013	0.025
Fungal culture	12	8	20	40.00		

fluorescence group and the 3' terminal is the quench group. Normally, these two groups are very close, with a FRET relationship. The fluorescence group can not erupt the fluorescence due to the quench. Therefore, only the 3' terminal fluorescence signal can be detected, but the 5' terminal fluorescence signal can not be detected. During PCR amplification, primers and probe combine with the template, and the probe is located between the upstream and downstream primers. When the amplification extends to the probe binding site, TaqDNA polymerase will hydrolyze the probe by using the 5' - 3' exonuclease activity, and the fluorescence group will be released and erupt the fluorescence. Therefore, once a new chain is synthesized, a probe will be hydrolyzed. With the increase of the product, the fluorescence signal is proportionally increased (Zhang et al., 2006; Bustin, 2000). MFRT-PCR could monitor multiple genes through several probes simultaneously.

Lai et al. (2004) considered that this technology had the following advantages: (i) Rapid; It only needs 3 min from PCR amplification to getting the result. (ii) Save time and effort; It can monitor more than 2 genes simultaneously, that is, it can detect more than two kinds of pathogens. If the automatic sample handling system is equipped, the DNA extraction and spotting of samples can be automatically finished within 5 min. Therefore, it only needs 30 - 40min from sample collection to getting the report. (iii) High sensitivity and specificity; the sensitivity and specificity were increased substantially due to the use of fluorescent probes and molecular luminescence technique. (iv) Low contamination; the man-made pollution is reduced, because the sample preparation, PCR amplification and results reading are automatically carried out in a closed system. (v) Quantitative detection could be carried out (Jerome et al., 2002).

Ct value of the amplification curve, also called the cycle threshold, represents the corresponding cycle number of the fluorescence signal which is firstly higher than the baseline level in the PCR process (or the cycle number when the signal in each reaction tube reaches the threshold). It is an important quantitative parameter in MFRT-PCR. All the fluorescence signals in each DNA amplification cycle are recorded, which was proportional to the amount of the amplification products. In the initial stage of the response, the template is more; the cycle number to the fluorescence signal threshold is fewer. The representative fluorescence signal of the threshold was significantly greater than the background signal. At that time, the required cycle number is Ct. It always appears in the exponential phase of the amplification. Therefore, the quantitative process is not affected by the reaction composition, but mainly depends on the original substrate concentration of the reaction system. At the diagnosis, the absolute copy number of the sample can be

accurately quantitated through the regression curve of the standard preparation. During the reaction process in the quantitative machine, the computer will automatically measure the Ct value of concentration of the positive standard which is the vertical axis. The logarithm of the positive standard template concentration is the X-axis, and the regression curve is established. Thus, the concentration (copy number) of the sample can be determined according to the positive Ct value (Yu et al., 2006). In our study, after MFRT-PCR of the standard preparation, the correlation coefficients of the standard curve were 0.996 and 0.994, indicating that the quantitative result was accurate and reliable.

In our study, MFRT-PCR was carried out by using the TaqMan probes of *C. albicans* and *A. flavus*. The positive rates of these two groups were 100% (10/10). Moreover, these two probes did not interfere each other and the copy number of each sample was high. The result also showed that the positive rates of the MFRT-PCR and fungal culture were 75.00% (15/20) and 40.00% (8/20), respectively, for 20 cases of suspected deep fungal infection patients. The sensitivity of these two methods was significantly different (x^2 = 5.013, P = 0.025), suggesting that the sensitivity of the former was higher than the latter. In the present study, it should be emphasized that 5 *C. albicans* infection and 3 *Aspergillus* infections were detected with fungal culture, while 11 *C. albicans* infection and 4 *Aspergillus* infections were detected with MFRT-PCR. Among them, 1 case was positive for both *C. albicans* infection and *Aspergillus* infection, but the quantitative number was low, suggesting that MFRT-PCR could detect the cases with low level infection. It is also applicable for the early infection and low-grade infection. The *C. krusei*, *C. glabrata*, *C. tropicalis* and *A. fumigatus* were not detected with MFRT-PCR, and the specificity was 100%. The main feature of MFRT-PCR is that it can not only quantitatively monitor the infected degree, but also simultaneously monitor two or more microorganisms in one reaction system and not interfere each other. Therefore, it can not only rapidly examine the samples, but also reduce the cost, which is beneficial to the patients. Simultaneously, the MFRT-PCR showed the good sensitivity and specificity in our study. Therefore, we can initially expand the "gold standard" of SLE accompanied with deep fungal infection in order to overcome the low sensitivity and long culture time of fungal culture. However, because the detection rate is proportional to the content of the microbiological samples, false-negative results may occur in the samples with low content.

In our study, we adopted the samples of SLE accompanied with deep fungus infection. We also can increase the number of the probes in order to getting the simultaneously quantitative detection of bacteria, virus and fungi.

REFERENCES

Bustin SA (2000). Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol., 25(2): 169-193.

Bon MA, van Oeveren-Dybicz A, van den Bergh FA (2000). Genotyping of HLA-B27 by real-time PCR without hybridization probes. Clin. Chem., 46(7): 1000-1002.

Fumiere O, Marien A, Fernandez Pierna JA, Baeten V, Berben G (2010). Development of a real-time PCR protocol for the species origin confirmation of isolated animal particles detected by NIRM. Food Addit. Contam. Part A Chem. Anal. Control Expo Risk Assess. 3: 1-10.

Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C (2001). An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. Methods. 25(4): 386-401.

Jerome KR, Huang ML, Wald A, Selke S, Corey L (2002). Quantitative stability of DNA after extended storage of clinical specimens as determined by real-time PCR. J. Clin. Microbiol., 40: 2609-2611.

Kang XP, Jiang T, Li YQ, Lin F, Liu H, Chang GH, Zhu QY, Qin ED, Qin CF, Yang YH (2010). A duplex real-time RT-PCR assay for detecting H5N1 avian influenza virus and pandemic H1N1 influenza virus. Virol. J., 7(1): 113.

Kato T, Mizokami M, Mukaide M, Orito E, Ohno T, Nakano T, Tanaka Y, Kato H, Sugauchi F, Ueda R, Hirashima N, Shimamatsu K, Kage M, Kojiro M (2000). Development of a TT virus DNA quantification system using real-time detection PCR. J. Clin. Microbiol., 38(1): 94-98.

Lai Wei, Chen CY, Su Xiangyang (2004). Detection of fever blisters virus with Multiplex fluorescent real-time PCR. Chin. J. Dermatol., 37(5): 273-275.

Malmström S, Berglin-Enquist I, Lindh M (2010). Novel method for genotyping hepatitis B virus on the basis of TaqMan real-time PCR. J. Clin. Microbiol., 48(4): 1105-1111.

Sun LD, Zhou ZG, Zeng K (2003). Investigation of fungal infection in systemic lupus erythematosus. J. Clin. Dermatol., 32(3): 146-147.

Wiwanitkit V (2010). TaqMan real-time PCR assay for Coccidioides. Med. Mycol., 48(4): 679.

Yu LX, Han SL, Miao Y (2006). CD95 and CD95L mRNA expression in the donee of liver transplantation with SYBR fluorescent real-time PCR. J. South. Med. Univ., 26(2): 185-188.

Zott K, Claisse O, Lucas P, Coulon J, Lonvaud-Funel A, Masneuf-Pomarede I (2010). Characterization of the yeast ecosystem in grape must and wine using real-time PCR. Food Microbiol., 27(5): 559-567.

Zhang CY, Cheng J, Li QS (2006). Development of real-time quantitativePCR. J. Jiangsu Univ. (Med. Edit.). 16(3): 268-271.