Development of a Real-Time Polymerase Chain Reaction (PCR) Assay using Hybridisation Probes to Detect the Fungus *Aspergillus*

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**ABSTRACT:**

We developed a real-time PCR assay on the LightCycler® instrument (Roche Molecular Systems) to detect the presence of *Aspergillus*, by detecting a 164bp fragment of the 18S ribosomal DNA (rDNA) gene. rDNA has traditionally been used as a phylogenetic marker in determining the relationship of one species or genus to another. Molecular identification of the 164bp amplicon was carried out using a pair of hybridisation probes, which rely on fluorescence resonance energy transfer to detect minute quantities of 18S rDNA. The assay was highly accurate (100% sensitivity; 100% specificity) in detecting various *Aspergillus* species in culture and five out of five samples containing 100\(\mu\)g of genomic DNA (corresponding to ~200 copies of the 18S rDNA gene in the starting material). It can in fact detect down to 10\(\mu\)g of DNA, with 60-80% consistency. There was no cross-reactivity with all 6 clinical *Candida* isolates tested. It achieved relatively less success (50% detection rate, n=6) when applied to detection of *Aspergillus* in paraffin-embedded tissues showing the characteristic morphologic appearance of septate, branched hyphae, used to identify fungi in tissue sections by histopathologists. This prototype assay has the potential to be a rapid (1h amplification and detection time), accurate and highly specific assay, which can be adapted for diagnosis and monitoring of invasive aspergillosis in clinical samples.

**INTRODUCTION:**

Fungal infections have been a major problem especially for the immuno-suppressed and immuno-compromised as they lack the ability to battle against such infections. Among fungal related infections, invasive aspergillosis (IA), caused by *Aspergillus* species is increasing at an alarming rate, with a 14-fold increase from 1980 to 1992 (Brookman and Denning, 2000). In addition, IA often causes complications in patients with chronic granulomatous disease (at rates as high as 40%), and in patients who had undergone organ transplants, with the highest IA rate at 26% for heart-lung transplantation. Cancer patients are not spared either, as 10% to 25% of leukaemia patients are diagnosed with IA, and mortality for such patients can be as high as 90%. Overall, patients with IA have mortality rate of 85%, in which most, if not all, died from IA (Lagte, 1999; Brookman and Denning, 2000).
Detection of the *Aspergillus* species is thus important in the diagnosis and monitoring of IA, and administration of proper treatments with antibiotics. Conventional methods that rely on culture methods with identifications based on microscopy are often prone to errors due to the similar diagnostic morphological features between *Aspergillus* and other genus, such as *Fusarium* (Hayden *et al*., 2000), and the lack of experiences in handling IA cases by the microbiologist.

Therefore, several methods had been devised, such as the usage of antibodies against the *Aspergillus* antigen galactomannan, and Polymerase Chain Reaction (PCR) with amplicon detection by gel electrophoresis. Nonetheless, such methods are limited by their accuracy, sensitivity, specificity and rapidity in detection of *Aspergillus*. An alternate solution lies in the usage of a LightCycler® (Roche Molecular Systems) that, unlike the conventional PCR that uses gel for detection of amplicon, uses a pair of hybridisation probes for detection after going through PCR. Upon binding of the pair of probes to the amplicon, the probes will be at close proximity to allow for fluorescence resonance energy transfer (FRET) to occur. The acceptor probe will absorb the fluorescence from the donor probe, and fluoresce at its unique wavelength for detection. Therefore, even minute quantities of DNA can be detected, and such a system is both quantitative and qualitative.

In view of the fact that IA cases can be caused by various *Aspergillus* species such as *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus niger*, detection at the genus level is crucial. The 18S ribosomal DNA (rDNA) gene thus served as the target for PCR amplification, as it is traditionally a phylogenetic marker that is generally conserved at the genus level.

This paper thus reports the development of a prototype (so termed, as it is our intention to further develop modifications of the current assay format reported herein) real-time PCR assay for the detection of the genus *Aspergillus*, through the detection of its 18S rDNA gene.

**MATERIALS AND METHODS:**

Briefly, the highly specific probes were designed firstly by alignment of the nucleotide sequences of the 18S rDNA of various *Aspergillus* species from Genbank to identify the conserved regions. Subsequently, the primer and probe pairs were chosen from a conserved region.

Amplification of 18S rDNA was carried out in 10µL reaction volume, with 0.5mM of primers, 0.2mM of hybridisation probes, 3mM of MgCl2, and 1X reaction buffer, at 95°C for 10s as denaturation phase, 60°C for 10s as annealing phase, and 72°C for 7s as extension phase. The three phases were repeated for 60 cycles.

To obtain the genomic DNA of *Aspergillus* species, the cultured hyphae mats were subjected to lyticase digestion to weaken the cell wall. Subsequent extraction procedures were as recommended in the protocol supplied with the extraction kit by QIAGEN, with slight modifications.

Calibration of the LightCycler® instrument was carried out using serial dilutions of the 164bp amplicon fragment, from 5.72 x 10⁹ copies down to 5.72 x 10² copies. The lower detection limit
of the assay was determined by serially diluting genomic DNA from 100pg (10^{-10} g) to 10fg (10^{-14} g). Reproducibility tests were further carried out with five replicates each at 100fg, 80fg, 40fg and 10fg of genomic DNA.

To evaluate the assay, genomic DNA from *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Candida albicans*, human host, and *Plasmodium* species were subjected to real-time PCR. In addition, six paraffin embedded tissues (PET) identified morphologically to be aspergillosis, and six actinomycetes containing PET were also used to evaluate the diagnostic sensitivity and specificity. Extraction of the genomic DNA from PET was as recommended in the QIAGEN extraction kit protocol, with the additional step of lyticase digestion.

**RESULTS AND DISCUSSION:**

**Calibration of LightCycler and Determination of Lower Detection Limit**

The standard calibration curve for the assay were determined by the crossing points, where the signal from the acceptor probe first increased exponentially, of various amplicon copy numbers. A gradient of −3.58 cycles per log concentration was achieved. This correlates to an efficiency of 1.9 amplifications per cycle compared to 2 amplifications per cycle in a perfect system, suggesting a high efficiency of the designed assay.

In terms of lower detection limits, studies using conventional PCR have a poorer sensitivity than the FRET-based system, which the real-time PCR is based on. In a research study by Melchers et al., only amplicons with a concentration not less than 1pg of genomic DNA were detected on a gel (1994). In comparison, the prototype assay we developed is much more sensitive as it can accurately detect 100fg of genomic DNA, and even down to 10fg at >60% of the time.

As for the minimal absolute numbers of *Aspergillus* cells, Loeffler et al. reported that more than 90 copies of 18S rDNA are expressed in each genome of *Aspergillus*. Another report, however, had obtained 10fg of genomic DNA from one to five *Aspergillus* colony forming unit (Skladny et al., 1999). The calibration results from our study showed that 100fg of genomic DNA correspond to about 200 copies of 18S rDNA gene, hence inferring that the number of *Aspergillus* cells detectable is between two and ten cells.

**Specificity of assay**

Usage of such hybridisation FRET probes underlies the high specificity in the prototype assay, as all the three common *Aspergillus* species (*Aspergillus fumigatus*, *Aspergillus niger*, and *Aspergillus flavus*) were detected but they did not cross-react with other organisms, such as *Candida* and actinomycetes, which could present with differential diagnostic conundrums.

**Validation of prototype real-time PCR assay**

Kawamura et al. had reported that detection of *Aspergillus* by the conventional PCR provided a much higher sensitivity than immunological tests such as double ELISA sandwich test and latex agglutination test (1999). The prototype real-time PCR assay also showed high sensitivity and specificity when detecting for cultured specimens. The sensitivity is 100% (*Aspergillus* species, n=3) while the specificity is also 100% (*Candida* isolates, n=6;
actinomycetes in PET blocks, n=6). Nonetheless, detection of *Aspergillus* species in paraffin embedded tissues proved to be difficult. The DNA isolation procedure might not have been 100% efficient, and thus the absolute amount of *Aspergillus* DNA may be below the critical mass threshold level, which may partially explain the detection rate of only 50% (three out of six samples) for *Aspergillus*-containing PET.

It must also be noted that species of *Aspergillus*, *Fusarium*, and *Pseudallescheria* all appear as septate, branched hyphae and the identity of such fungus could be misidentified initially in the PET specimens which were subjected to real-time PCR (Hayden *et al.*, 2003).

**CONCLUSION:**

This paper reports the development of a prototype quantitative real-time PCR-based assay for the detection of *Aspergillus*. The assay showed high sensitivity and specificity (100% for both), which is comparable or superior to existing qualitative assays by other authors. In addition, it is capable of detecting as low as 10 copies (10 fg) of target DNA, and rapidly, which is crucial in reducing morbidity and mortality rates due to IA. The potential of the prototype assay could well be extended to monitoring therapeutic efficacy of different anti-fungal agents.

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