Evaluation of Galactomannan Enzyme Immunoassay and Quantitative Real-Time PCR for the Diagnosis of Invasive Pulmonary Aspergillosis in a Rat Model

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Introduction

The prevalence of invasive fungal infections and consequent mortality has increased throughout the last two decades, and the reported mortality from an epidemiological study covering the period from 2002 to 2006 was 28.6% [7]. According to the TRANSNET database, half of extensive fungal infections are caused by Aspergillus sp. [4]. Most fungal infections occur in immunodeficient individuals, such as transplant recipients or leukemia patients who are receiving chemotherapy. Pathological exacerbations of lung infections and the inability to diagnose fungal infections are the major causes of death in these patients [3, 22]. The early diagnosis of invasive fungal infections is critical for rapid treatment [22], but the lack of sensitive and specific clinical symptoms and radiological patterns for invasive aspergillosis (IA) hinders early diagnosis. Traditional histopathological examination and fungal culture rely on invasive procedures, are relatively insensitive, and not are commonly used in clinical diagnosis owing to the challenges of sampling pulmonary fluids or tissues from critically ill patients. Although the examination of bronchoalveolar lavage (BAL) fluid yields a higher detection rate than the examination of blood or serum [1, 6, 11], obtaining BAL...
fluid is invasive, and many patients with IA have other severe diseases that limit BAL collection. Consensus on the most reliable assays to detect IA from minimally or noninvasive samples has not been reached, and is a major topic of current research [18, 28].

Galactomannan (GM) is a cell wall component of Aspergillus, and its production increases during Aspergillus growth. Assays that detect fungal antigens such as GM by enzyme-linked immunosorbent assay (GM assay) or Aspergillus DNA by polymerase chain reaction (PCR) are emerging diagnostic methods; however, their specificity and sensitivity require additional characterization and refinement. The sensitivity of the GM assay ranges from 60% to 100% for infected Aspergillus samples, and the specificity ranges from 80% to 100% [2, 5, 8, 9, 24]. The cut-off value has a significant impact on diagnosis because of cross-reactivity [17, 18, 23, 24, 26]. Furthermore, treatment with antifungal therapies decreases the fungal load and reduces the GM concentration, which can fall below the detectable limit of the GM assay [15]. Given the advantage of high sensitivity, a PCR assay coupled with an ELISA assay (PCR-ELISA) has been used for detection of Aspergillus [19, 20]. However, poor specificity has been noted, due to several interference factors, including environmental contamination [19]. In addition, the PCR-ELISA assay is more cumbersome for clinical laboratories [6].

Recently, a quantitative real-time PCR (qPCR) assay that may overcome the shortcomings of other methods was developed [14, 18]. qRT-PCR analysis of serum samples of patients with hematological malignancies at risk for IA was shown to have a sensitivity of 72.7% [6]. A commercially available qRT-PCR for the detection of Aspergillus DNA (MycAssay Aspergillus) has shown a sensitivity of 60–70% and a specificity of 95% for the detection of IA [25]. However, a study by Scotter and Chambers [19] indicated that GM testings by ELISA and the PCR-ELISA were more capable of early detection of fungal infection than RT-PCR examination alone.

The aim of this study was to compare the sensitivity and specificity of the GM assay with that of the Aspergillus-specific nucleic acid qRT-PCR assay in a rat model of pulmonary IA.

Materials and Methods

Aspergillus fumigatus Preparation

Lyophilized Aspergillus fumigatus (A. fumigatus) was recovered in 0.5 ml of sterile broth, and cultured in Sabouraud agar medium at 37°C for 48 h, and subsequently at 30°C for 3–5 days. Spores were eluted from the agar surface with 10 ml of PBS containing 0.05% Tween-80. The suspension was filtered through eight layers of sterile gauze to remove hyphae. The spore suspension was then transferred to a 15 ml tube and centrifuged at 10,000 g for 15 min. The supernatant was discarded, and the pellet containing spores was resuspended in normal saline. Spore count was determined on a blood count plate, and spore viability was determined by culture of serial dilutions of the spore suspension. The concentration was adjusted to 8 × 10⁵ spores/ml.

Induction of Pulmonary Aspergillosis in Rats

Neutropenic rats were infected with Aspergillus as described by Zhang et al. [27]. Briefly, male Sprague-Dawley rats (specific pathogen free; 8 weeks old; weight, 200 ± 20 g) were housed with food and water ad libitum according to animal care guidelines. Rats received one of four treatments: immunosuppression and A. fumigatus infection (n = 48); immunosuppression (no infection, n = 6); infection (no immunosuppression, n = 6); and vehicle control (no immunosuppression, no infection, n = 12). Persistent immunosuppression was induced in the indicated groups by intraperitoneal injection of cyclophosphamide, as described by Leenders et al. [10]. The optimal dosage of cyclophosphamide (CP), determined by a preliminary experiment, involved three injections: CP (50 mg/kg) and CP (40 mg/kg) were injected i.p. 5 days and 1 day prior to the Aspergillus spore suspension inoculation; and CP injection (30 mg/kg) was performed on day 3 after inoculation. Control groups were injected with an equivalent volume of normal saline. After CP treatment, all rats were consistently kept in a clean environment, and injected with levofloxacin, 10 mg/kg/day.

After anesthesia (chloral hydrate (3.5 ml/kg i.p.) and intubation, the immunosuppressed and normal rats of the indicated groups were infected with A. fumigatus (8 × 10⁵ spores) by injecting 0.1 ml into each endotracheal tube. Rats were kept erect and rotated for 30 sec, ensuring that the inoculation entered the trachea and was distributed evenly in both lungs. Rats that received both immunosuppression and infection (n = 48) were sacrificed on days 1, 3, 5, and 7 after inoculation, and were referred to as group 1, 2, 3, and 4, respectively (n = 12/group). Whole blood (4 ml) was collected by heart puncture from each individual rat: 3 ml of whole blood was used for DNA isolation, and serum was prepared to measure the GM concentrations using the ELISA method, as per the manufacturer’s instructions. Lung tissue was collected for immunohistochemical analysis.

Isolation of A. fumigatus DNA from Blood

A. fumigatus DNA was isolated from whole blood, as described previously [13]. Briefly, 3 ml of whole blood was treated with 1 ml of EDTA anticoagulant, lysed in 1 ml of erythrocyte lysis buffer (0.01 mol/l Tris-HCl (pH 7.6), 0.01 mol/l NaCl, 0.005 mol/l MgCl₂), mixed thoroughly, and further treated with 1 ml of lysis buffer twice, allowing extensive erythrocyte lysis. After centrifugation at
8,000 ×g for 10 min, the pellet was washed with normal saline; the physical method of grinding using a mortar and pestle, similar to bead beating, broke the cell walls of the fungi and released fungal DNA, with an efficiency of approximately 96.8%. The pellet was resuspended in 2 ml, and treated with 100 µl of lysis buffer (20 mM Tris-HCl, 2.0 mM EDTA, 1.0% Triton X-100, 2 mg/ml proteinase K), and subsequently 50 µl of lysozyme at a concentration of 50 µg/ml. The lysate was incubated at 60°C for 60 min, extracted with an equivalent volume of phenol, then chloroform, and isoamyl alcohol. A. fumigatus DNA was ethanol precipitated, dissolved in Tris-EDTA buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA), and stored at −20°C for reserve.

Polymerase Chain Reaction and Product Analysis

Specific primers were based on the target mRNA sequence in GenBank, harboring the CDS region of the mitochondrial translation optimization gene Motl of A. fumigatus. Primers were designed using Primer Express 2.0 software: forward primer, 5’-tttcatcaccgaagctcttt-3’; reverse primer, 5’-gaattcctcgagggagcgtcct-3’; probe, 5’-FAM-cagttgtgatgacgacacgcccagt-TAMRA-3’. Primers were synthesized using the ABI 3900 high-throughput DNA synthesizer. To determine the specificity of the qRT-PCR, the aforementioned primers were assessed for their ability to amplify DNA from A. niger, A. flavus, A. terreus, Candida albicans, Cryptococcus neoformans, Staphylococcus aureus, and Pseudomonas aeruginosa [12].

The qRT-PCR (50 µl) included 10 µl of 5× reaction buffer (10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2 mM MgCl2), 10 pmol of each primer, 2 µl of dNTPs (10 mM), 3U of Taq DNA polymerase, and 4 µl of DNA or positive standard. Reaction was performed as follows: 93°C for 3 min, and 40 cycles of 93°C for 30 sec, 55°C for 45 sec. Real-time PCR was carried out in an automated fluorescent quantitative PCR cycler (ABI 7500), and the amplification curve was analyzed based on the exponential amplification and Ct value (cycle threshold). The Ct was defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e., exceed background level). The Ct value was dose-dependent on the positive standard.

The standard curve quantification method used the target gene synthesized in Sangon (Shanghai). Ten-fold serial dilutions of the positive recombinant plasmid standard (optical density (OD) 260/280 >1.8) were utilized in the qRT-PCR amplification following the optimal procedures. Distilled water was used as a negative control. The limit of detection (LOD) was measured according to the amplification curve derived from qRT-PCR. The correlation between Ct value and DNA copy was Ct = -3.347424 × log copy number + 35.885406.

GM Antigen Detection (GM Assay)

GM concentrations in test serum, standardized controls, or negative control were measured in triplicate (100 µl each) by using the Platelia Aspergillus kit (Bio-Rad Corporation, France), following the manufacturer’s instructions with a modification in cut-off values recommended by Becker et al. [2].

Histopathological Examination

Rats that received both cyclophosphamide and infection were sacrificed on 1, 3, 5, and 7 days after A. fumigatus inoculation (n = 12 each group). Rats in the other groups were sacrificed on day 7. Blood samples and right lung tissue were used for fungal culture as described above. The left lung tissue was fixed in 10% formalin and embedded in paraffin. The paraffin sections were primarily stained with hematoxylin & eosin (HE), and further with periodic acid-Schiff stain, followed by histopathological examination.

Statistical Analysis

The values of the GM concentration for each group are reported as the mean ± standard deviation. The Wilcoxon rank sum test was used to analyze the difference between each set of two groups. The PCR results were analyzed using the Fisher’s exact test. A receiver operating characteristic (ROC) curve was created with the statistical software used for all analyses (SPSS ver. 16.0). A value of p < 0.05 was considered to indicate statistical significance.

Results

Histopathology of Pulmonary A. fumigatus Infection

Immunosuppressed, infected rats showed progressive accumulation of hyphae in the alveoli (Fig. 1). Many Aspergillus spores and mild inflammation were found in the lung tissue of the neutropenic rats on the 1st day post-

![Histopathological analysis](image-url)

>Fig. 1. Histopathological analysis. Lung tissues were harvested from immunosuppressed, infected rats on days 1 (A), 3 (B), 5 (C), and 7 (D) (100× magnification), and from the nonimmunosuppressed and not infected control group on day 7 (E) (400× magnification).
infection (Fig. 1A). On the 3rd day, the alveoli exhibited accumulated spores, a small presence of hyphae, hyperplasia of alveolar epithelial cells, and broadened alveolar septa. Cellulose exudate and hemorrhage in the alveolar spaces were also observed (Fig. 1B). On the 5th day post-infection, abnormal morphology of the alveolar structures was apparent, and the spores had bloomed: hollow, colorless, and acute angle-branching hyphae and granuloma were present (Fig. 1C). Clear exudate was located in the alveolar spaces, and severe hemorrhage was evident in the capillaries. The alveolar septa had broadened, and part of the alveolar structure was damaged. On the 7th day, the aforementioned characteristics had become more apparent and the alveolar structure was no longer clear (Fig. 1D). The alveoli of the immunosuppressed but not infected control group had normal morphology (Fig. 1E). No apparent inflammation was noted in nonimmunosuppressed, infected rats.

Detection of *A. fumigatus* DNA Isolated from Immunosuppressed, Infected Rats

*A. fumigatus* target sequences were not detected in the two uninfected control groups; the immunosuppression and no infection group (*n* = 6) or in the vehicle control group (no immunosuppression, no infection, *n* = 12). The *A. fumigatus* DNA sequence was not detected in the day 1 and day 3 blood samples from the immunosuppressed, infected rats, suggesting that the lag period of the infection extended to at least day 3. The *A. fumigatus* DNA sequence was detected in 50% (6/12) of the day 5 samples and 66.7% (8/12) of the day 7 samples, indicating the spread of the *A. fumigatus* infection in the immunosuppressed, infected rats.

Amplification curves and quantitative analyses showed that the DNA content ranged from $5 \times 10^2$ to $5.77 \times 10^3$ copies/µl of blood (data not shown). The lower limit for qRT-PCR detection was 100 copies/µl of blood, and was determined according to the amplification curve derived from qRT-PCR. Only samples from infected animals yielded positive RT-PCR curves. ROC curve analysis indicated a Ct cut-off value of 15.35, and the area under the curve (AUC) was 0.627 (Fig. 2).

The specificity of the primers and probe was examined in PCRs that used a DNA template from *A. niger*, *A. flavus*, *A. terreus*, *Candida albicans*, *Cryptococcus neoformans*, *Staphylococcus aureus*, or *Pseudomonas aeruginosa*. These PCRs yielded negative results, which indicated that the primer set for *A. fumigatus* showed high specificity.

Determination of GM by ELISA

A commercial kit was utilized for the determination of GM, and the mean concentrations for each group are shown in Table 1. As expected, the concentration of GM increased from day 1 to day 7, in agreement with the infiltration observed in the histopathological specimens. The GM assay detected antigen in only some of the infected rats.

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**Fig. 2.** ROC curve of qRT-PCR assay.
At a Ct value of 15.35, the sensitivity was 25.5%, the specificity was 100%, and the area under curve was 0.627.

**Fig. 3.** ROC curve of the GM assay.
At a cut-off 1.40, the sensitivity was 79.2%, the specificity was 100%, and the area under curve was 0.919.

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A. fumigatus Detection by ELISA vs qRT-PCR

**Table 1.** qRT-PCR detection of Mto1 gene copy number and serum GM levels.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number GM positive/Total</th>
<th>Serum GM level (index)</th>
<th>qPCR of blood (copies/reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Infection and immunosuppression</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1 (group 1)</td>
<td>5/12</td>
<td>1.236 ± 0.169</td>
<td>ND*</td>
</tr>
<tr>
<td>Day 3 (group 2)</td>
<td>9/12</td>
<td>1.889 ± 0.247</td>
<td>ND</td>
</tr>
<tr>
<td>Day 5 (group 3)</td>
<td>12/12</td>
<td>2.548 ± 0.218</td>
<td>35.29 ± 31.25</td>
</tr>
<tr>
<td>Day 7 (group 4)</td>
<td>12/12</td>
<td>3.520 ± 0.215</td>
<td>1,183.69 ± 859.62</td>
</tr>
<tr>
<td><strong>Control groups at Day 7</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunosuppression/no infection</td>
<td>0/6</td>
<td>0.857 ± 0.103</td>
<td>ND</td>
</tr>
<tr>
<td>No immunosuppression/infection</td>
<td>0/6</td>
<td>0.683 ± 0.130</td>
<td>ND</td>
</tr>
<tr>
<td>Vehicle control: No immunosuppression/no infection</td>
<td>0/12</td>
<td>0.600 ± 0.109</td>
<td>ND</td>
</tr>
</tbody>
</table>

*ND = could not be detected.

Wilcoxon rank sum test showed that the serum GM levels in the immunosuppressed infected animals at days 1, 3, 5, and 7 were significantly different from the control groups (p < 0.01). A significant difference was also present between group 1 and group 4 (p < 0.01). No significant difference was observed in the GM levels between the three control groups (all p > 0.05).

Discussion

In this study, the efficacies of the GM assay and qRT-PCR for the detection of *Aspergillus* infection were evaluated in a well-established rat model of pulmonary IA. The results showed that although both assays were 100% specific for the diagnosis of IA, the GM assay exhibited much greater sensitivity and allowed for earlier detection.

Serum-based assays for the diagnosis of *Aspergillus* infection have been sought for at least four decades, but inadequate sensitivity has restricted the general application of many methods [18]. A commercial ELISA method measures GM antigen at concentrations as low as 0.5–1 ng/ml, depending on the cut-off value [13, 27]. Our cut-off value of 1.4 provided a sensitivity of 79.2% for days 1–7 infected samples in this immunosuppressed rat model. Using an *A. fumigatus*-infected guinea pig model, McCulloch et al. [15] found that the GM assay was able to detect *A. fumigatus* infection in samples from 0/3 animals on day 1, 1/3 on day 2, and 3/3 on days 3–5, for an overall sensitivity of 67%. Lengerova et al. [11] observed a 100% sensitivity in BAL fluids in an IA rat model, but only a 26% sensitivity in serum samples (1/5 on day 3, 0/5 on day 5, 3/5 on day 7). Becker et al. [2] found that the GM assay detected GM in 8% of day 1 samples in an IA rat model, 89% of day 3 samples, and reached 100% by day 7. In comparison, our results showed that GM was detected in the sera of 5 of 12 samples on day 1, 9 of 12 samples on day 2, and all samples on days 3–7, which was consistent with other reports [15]. The commercially available MycAssay *Aspergillus* DNA assay and an “in house” qRT-PCR assay have shown promise in the clinical diagnosis of IA via testing BAL fluid samples [21]. Compared with conventional PCR, qRT-PCR lowers the risk of cross-contamination by using a sealed tube during amplification and detection. The fluorescence signal generation depends on both probe-template recognition and amplification of template, ensuring the high specificity of the qRT-PCR method. Although *Aspergillus* sp. DNA is usually detected at a higher frequency in BAL samples [21], Hadrich et al. [6] observed that RT-PCR and PCR-ELISA assays exhibited higher sensitivities in serum samples than in BAL samples, with sensitivities ranging from 64%–94%.

In our rat model, the sensitivity of the qRT-PCR was 25% with whole blood samples harvested from infected, immunosuppressed rats on days 1–7, which was similar to the 26% sensitivity using serum samples from a guinea pig IA model (days 1–7) reported by Lengerova et al. [11]. Likewise, Becker et al. [2] found that in an IA rat model, PCR did not detect *Aspergillus* DNA from day 1 or 2 serum samples, but the sensitivity was improved from 20% on day 3 to 40% on day 7, and the authors concluded that the sensitivity may be related to the methodology. One obvious difference between humans and animal models is that, in most cases, humans will have sought testing...
because of the presence of symptoms, suggesting a more fulminant Aspergillus infection at the time of sampling than the early time points in animal models. Presumably, the longer incubation period would favor Aspergillus replication, and may promote alveolar damage and seepage of more Aspergillus spores into the blood.

In this study, analysis of the qRT-PCR results showed that an AUC of 0.627 produced the maximum specificity, and although highly consistent with the pathological changes in lung tissue, the qRT-PCR method was not sufficiently sensitive (25.9%) to be solely relied on for early diagnosis. In contrast, the AUC of the GM assay was significantly higher (0.919), and was able to detect Aspergillus in 79% of days 1–7 samples. Interestingly, Torelli et al. [21] described a cohort of patients with suspected IA, and >90% of patients with BAL samples that were positive for Aspergillus DNA by MycAssay and RT-PCR also had GM-positive BAL samples. Together, it seems that the GM assay and qRT-PCR both are capable for the early detection of Aspergillus infection, but further study for establishing a gold standard is warranted [16].

In summary, the GM assay detected Aspergillus GM antigen as early as day 1 in some samples, on day 3 in most samples, and on day 5 in all samples, whereas qRT-PCR analysis detected only half of the infected samples by day 5. Thus, the GM assay showed superior diagnostic sensitivity in the early days post-infection in our pulmonary IA rat model compared with qRT-PCR analysis.

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References


