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Environmental Investigations and Molecular Typing of *Aspergillus* in a Chinese Hospital

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Abstract

Invasive fungal infections due to *Aspergillus* species have become a major cause of morbidity and mortality among immunocompromised patients. In order to determine the possible relationship between environmental contamination by *Aspergillus* and the occurrence of invasive aspergillosis, a 1-year prospective study was carried out in a tertiary hospital in China. Air, surface, and tap water sampling was performed twice monthly at the bone marrow transplant (BMT) department, intensive care unit (ICU), neurosurgery intensive care unit (NICU), and outdoors. Nose, pharynx, and sputum samples were collected from high-risk patients. Isolates of *Aspergillus* from the environment and patients were genotyped by random amplification of polymorphic DNA (RAPD) assay to investigate the origin of infection. Mean total *Aspergillus* count was 7.73, 8.94, 13.19, and 17.32 cfu/m³ in the BMT department, ICU, NICU, and outdoors, respectively. RAPD analysis by R108 primer demonstrated that strains isolated from patients in NICU were identical to the environmental strain. Strains isolated from patients in ICU differed from the environmental strain. *Aspergillus* contamination was found in the BMT department, NICU, and ICU. Clinical and environmental strains from NICU had identical genotypes. These findings suggest that *Aspergillus* is found in the hospital environment including the air, surface, and tap water. The genotypes of *Aspergillus* were identical from patients and the environment, suggesting that clinical infection may originate from the hospital environment.

Keywords (separated by ‘-’) *Aspergillus*- Aspergillosis - Environmental contamination - Hospitals
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Environmental Investigations and Molecular Typing of Aspergillus in a Chinese Hospital

Jun-hong Ao • Zhen-feng Hao • He Zhu • liang Wen • Rong-ya Yang

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Abstract Invasive fungal infections due to Aspergillus species have become a major cause of morbidity and mortality among immunocompromised patients. In order to determine the possible relationship between environmental contamination by Aspergillus and the occurrence of invasive aspergillosis, a 1-year prospective study was carried out in a tertiary hospital in China. Air, surface, and tap water sampling was performed twice monthly at the bone marrow transplant (BMT) department, intensive care unit (ICU), neurosurgery intensive care unit (NICU), and outdoors. Nose, pharynx, and sputum samples were collected from high-risk patients. Isolates of Aspergillus from the environment and patients were genotyped by random amplification of polymorphic DNA (RAPD) assay to investigate the origin of infection. Mean total Aspergillus count was 7.73, 8.94, 13.19, and 17.32 cfu/m³ in the BMT department, ICU, NICU, and outdoors, respectively. RAPD analysis by R108 primer demonstrated that strains isolated from patients in NICU were identical to the environmental strain. Aspergillus contamination was found in the BMT department, NICU, and ICU. Clinical and environmental strains from NICU had identical genotypes. These findings suggest that Aspergillus is found in the hospital environment including the air, surface, and tap water. The genotypes of Aspergillus were identical from patients and the environment, suggesting that clinical infection may originate from the hospital environment.

Keywords Aspergillus Aspergillosis Environmental contamination Hospitals

Introduction

Aspergillus species are commonly found in soil, decaying organic matter, dust and air and are ubiquitous filamentous fungi that can cause severe opportunistic human diseases. In immunocompromised hosts, the inhalation of conidia may provoke pulmonary, invasive, and disseminated infection, associated with high mortality. During recent years, research has focused on the epidemiology of invasive fungal infections and the prevalence of fungi in the hospital environment [1]. Infecting fungal strains can be derived from the hospital environment. Contamination of the hospital environment by fungal spores is frequently implicated as the cause of aspergillosis in
hematological wards or intensive care units (ICUs). However, no consensus or overall conclusions have been reached. The incidence of invasive fungal infections due to Aspergillus species has increased dramatically during the past two decades, and despite all diagnostic and therapeutic efforts, outcome is often fatal [2]. The molecular typing of Aspergillus has proven useful in many epidemiological situations. One of the most widely used genotyping methods for Aspergillus is the random amplification of polymorphic DNA (RAPD) PCR, a technically relatively simple and rapid procedure [3, 4]. In this study, the source of Aspergillus strains collected prospectively over a 12-month period from environmental and clinical samples was investigated. Another objective was to determine the possible relationship between environmental contamination by Aspergillus and the occurrence of invasive aspergillosis (IA).

Materials and Methods

The study was performed from January to December 2009 at a 1,600-bed tertiary care teaching hospital in Northern China. The hospital has 2,800 staff and consists of four inpatient department buildings of different ages. Three departments with high-risk patients were selected for the study. The ICU is on the eleventh floor, and the neurosurgical ICU (NICU) is on the fifth floor. Both ICUs have central air conditioning, and air exchange occurred between the rooms and outdoors via the windows. The bone marrow transplant (BMT) unit is on the fourth floor, and air is mechanically imported into the room via a high-efficiency particulate air filter. The three departments are all located in the same building, which was constructed in 1997. Air, surfaces, and tap water were sampled between 09:00 and 12:00 h twice monthly over a 12-month period.

Air Sampling

Air sampling was performed with the LWC-I centrifugal sampler (Liaoyang Application Technology Corporation, China), which inoculated 1,600 L of air onto Sabouraud’s dextrose agar (SDA) culture medium. Air samples were obtained approximately 1.5 m above the floor and for about 2 min. The air in the ward, corridor, and an outdoor site was selected for sampling. One outdoor site, the entrance of the hospital building, was investigated to compare the measurements in the hospital with those outside. During each sampling, we recorded the environmental conditions (humidity and temperature) and special events, such as the presence of many people, including visitors, healthcare staff, and cleaning crew.

Surface Sampling

Surface sampling was also performed with the LWC-I centrifugal sampler. Surface samples were taken along a surface for about 1 min. Air-conditioning units, nursing consoles, and water tap surface were selected for sampling.

Water Sampling

Five hundred milliliters of tap water from the wards was collected in sterile containers and filtered through filter units with a pore diameter of 0.45 μm (Sigma, St Louis, MO, USA). The filters were then cultured on SDA plates for 24 h.

Patient Sampling

Patients hospitalized in the BMT department, ICU, and NICU were screened for nasal, sputum, and bronchoalveolar lavage fluid twice weekly. Patients for monitoring included: (1) those who were treated in hospital for ≥48 h; (2) patients with high risk for Aspergillus infection (organ transplantation, long-term treatment with multiple broad-spectrum antibiotics, immunosuppressants and/or glucocorticoids, indwelling catheter, tracheostomy and assisted ventilation, and neutropenia); (3) body temperature ≤38 C; (4) cough, expectoration, dyspnea, hemoptysis or pleural friction rub; and (5) chest imaging revealed newly occurring foci. The nasal and throat secretions, sputum, and bronchoalveolar lavage fluid were collected for culture. Swabs were taken for inoculation of SAB plates and broth and processed further as described above. All suspected IA cases were reviewed and classified as proven, probable, and possible according to European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) criteria [5]. Any clinical Aspergillus isolates obtained from patients were collected and subjected to molecular typing.
Fungal Identification

All samples were incubated at 26 C and inspected daily for growth (colony and colonial morphology) for 3 days. The environmental fungal load was calculated after 3 days. Colonies were then inoculated onto separate potato dextrose agar (PDA) plates, and the isolates were identified according to their macroscopic and microscopic morphological characteristics.

Genomic DNA Extraction

Isolates were subcultivated and cultured on PDA slants at 26 C for 7-14 days. Colonies were watered by NaCl and filtrated by 8-layer carbasus to yield conidia. Conidia were adjusted to 10^9 cells/ml, suspended in 300 l DNA extraction buffer [2 % Tritox-100, 1 % SDS, 100 mM NaCl, 10 mM Tris-HCl (pH 8.0)], vortexed with 200 l glass beads for 15 min, swung with coding SAT NaCl (6 M) for 15 s, and centrifuged for 10 min at 10,000 r/min. Isovolumic phenol/chloroform was added to the supernatant, which was shaken for 5 min and centrifuged for 10 min at 12,000 r/min. Isovolumic isopropanol was then added to the supernatant, which was centrifuged for 15 min at 10,000 r/min, rinsed with 40 % ethanol for 1 min, and centrifuged for 5 min at 10,000 r/min. Deposition were adding TE and conserving in -20 C.

RAPD Assay

RAPD assay was performed using four primers: R108 (GTATTGCCCCT), OPAX (AGTGCACACC), OPQ6 (GAGCGCCTrG), and R151 (GCTGTAGTGT). PCR was carried out in 25 l containing 2.5 l of 109 reaction buffer, 1 l 100 pmol primer, 2 l dNTP (2.5 mmol/l), 0.5 l Taq DNA polymerase (2 U/l), and 0.5 l 11 genomic DNA (10 ng/l). After denaturing at 94 C for 5 min, the thermal cycle conditions were 94 C for 30 s, 35 C for 30 s, and 72 C for 75 s for 44 cycles, followed by final extension at 72 C for 5 min. PCR product was separated on a 1.5 % agarose gel, stained with ethidium bromide, and photographed.

Statistical Analysis

The relative position of the reaction strap was determined first. On the same molecular weight, the reaction having strap as “1”, no-reaction strap as “0”. Reaction strap of all strain was diverting data block composing with “1” and “0”. The distance of the strain was calculated, and a clustering dendrogram was drawn to estimate the relationship between the genotypes of different strains, using SPSS version 12.0.

Results

Monitoring of Aspergillus Infection in Ward Environment

A total of 982 environmental samples were collected: 336 air samples, 478 surface samples, and 168 water samples. Fluctuations in the Aspergillus isolates from the air samples over the 12 months are shown in Fig. 1. Aspergillus was found in the air samples from the hospital, and the levels fluctuated during the year. The highest values were recorded during May-August and were lower in the winter and higher in spring and summer. From January to March, Aspergillus load outside the hospital was generally low, ranging from 3,13 to 9.38 cfu/m^3. After March, an increase was noted. The highest Aspergillus load outside the hospital was recorded in July (46.88 cfu/m^3). Similar to outside, from January to April inside the hospital had a generally low Aspergillus load and an increase was noted after April. The highest Aspergillus load was recorded in the NICU in May (35.94 cfu/m^3), ICU in July (21.88 cfu/m^3), and BMT department in June (21.88 cfu/m^3). The mean total Aspergillus load was 7.73, 8.94, 13.19, and 17.32 cfu/m^3 in the BMT department, ICU, NICU, and outdoors, respectively. The 5 most prevalent fungi collected from air were Aspergillus flavus, Aspergillus niger, Aspergillus fumigatus, Aspergillus versicolor, and Aspergillus clavatus (Table 1). For the surface samples, the highest Aspergillus concentration was found in air collected from the air-conditioning outlet of the ICU. The detection rate of A. niger was the highest (41 %) in samples collected from water in the BMT department. The detection rate of A. fumigatus (26 %) was the highest in samples from water from the NICU, but that of A. niger was relatively low (12 %). The detection rate of A. flavus (65 %) was the highest in water samples from the central ICU, but that of A. niger was as low as 5 %.
Discussion

In the present study, 982 environmental samples were collected: 336 from the air, 478 from surfaces, and 168 from water. A total of 224 strains of A. flavus, 117 of A. niger, and 62 of A. fumigatus were identified. This suggested that ward environment was contaminated by Aspergillus to different extents. The mean total Aspergillus load was 7.73, 8.94, 13.19, and 17.32 cfu/m$^3$ in the BMT department, ICU, NICU, and outdoors, respectively. The 5 most prevalent fungi collected from air were A. flavus, A. niger, A. fumigatus, A. versicolor, and A. clavatus. The detection rate of A. niger was highest (41 %) in water samples collected from the BMT department. The detection rate of A. fumigatus (26 %) was highest in water samples from the NICU, but that of A. niger was relatively low (12 %). The detection rate of A. flavus (65 %) was highest in water samples from the central ICU, but that of A. niger was as low as 5 %.

Besides surveillance of environmental air, 35 patients with a high risk of infection were also surveyed, who comprised 10 patients with BMT, 12 with tracheotomy in the NICU, and 13 in the central ICU. These patients had risk factors for infection such as organ transplantation, long-term treatment with broad-spectrum antibiotics, immunosuppressive agents or glucocorticoids, undergoing surgery, with tracheotomy and concomitant-assisted ventilation. The nasal and throat secretions and sputum were collected for culture of fungi. In addition, symptoms and signs were examined, and chest X ray and/or computed tomography were performed. In 5 patients, a total of 33 strains of A. flavus and 3 strains of A. fumigatus were identified. These 5 patients were diagnosed as highly suspicious for IA according to the criteria developed by EORTC/MSG.

In the RAPD assay, a randomly synthesized single oligonucleotide was used for amplification of DNA in target cells by PCR, and the PCR products were subjected to gel electrophoresis, and the size and number of DNAs were then analyzed, aiming to compare the difference in target gene. This technique has been used in the identification, classification, and epidemiological study of yeasts and filamentous fungi. Lasker et al. [6] applied primers R108, R151, and UBC90 to the RAPD of 47 strains of A. fumigatus and showed that primer R108 had the favorable ability of identification (D = 0.902). Anderson et al. [7] and...
Mondon [8] applied primers OPAX and OPQ6 to the RAPD analysis of *A. fumigatus*, and PCR products were shown to be clear. Thus, we used primers R108, OPAX, OPQ6, and R151 in our RAPD analysis, but only primer R108 could clearly display the polymorphisms of *A. flavus*. PCR with this primer had stable and clear bands and the bands for the PCR products of the other three primers were blurred, unstable, and difficult to discern. Thus, primer R108 was used for RAPD analysis of 152 strains of *A. flavus*.

Of note, the bands for the PCR products with primer R108 were slightly different from those in previous studies in which annealing temperature was 36°C, and 2.5 mM magnesium solution was used. In the...
present study, the annealing temperature was 35°C, and 2.0 mM magnesium solution was used. This discrepancy may be attributed to the difference in the species of *Aspergillus*. In the present study, *A. flavus* was analyzed, but *A. flavus* was investigated in previous studies. PCR with R108 generated 2-9 bands, and the minimal segment was about 300bp, and the maximal segment was 2 kb. The number of bands and the size of segments differed among *Aspergillus* species. Even among some *Aspergillus* species that had identical bands, there were differences in several segments.
In Aspergillus infection, environmental factors also play an important role in addition to host factors. Contamination of the hospital environment by fungal spores has frequently been implicated as the cause of aspergillosis in hematological wards or ICUs. After an outbreak of sternal surgical-site infection with A. flavus following cardiac surgery, Heinemann et al. [9] selected primers ERIC-1 and BG-2 to analyze A. flavus from the operation site and bronchoalveolar lavage fluid by RAPD. They found that A. flavus isolated from the patients and the hospital environment had the same genotype, which confirmed that the infection originated from the hospital environment.

Menotti et al. [10] selected primers R108, OPAX, and OPQ6 to analyze A. fumigatus from the environment and patients with IA by RAPD. A. fumigatus was isolated from the sputum and bronchoalveolar lavage. A. fumigatus from the patients and the environment had the same genotype, confirming the source of infection as the hospital environment. Our results provide a preliminary understanding of Aspergillus contamination and distribution in the BMT department, NICU, and central ICU. In addition, RAPD was also performed to genotype the A. flavus from the air samples and the patients. We demonstrated that A. flavus from 2 patients in the NICU was identical in genotype to those from the ward air. This suggests that A. fumigatus infection can be attributed to fungi in the ward air. The A. flavus from 2 patients in the central ICU also had the same genotype, suggesting potential cross-infection of A. flavus in the ward.

Acknowledgments This study was supported by the grants from National Natural Science Foundation of China (81201236 and 81071304) and Special Research fund of Capital Health Development (2011-5021-04).

Table 1 Different fungal species in environmental and patient samples

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References