Original Article

Anti-fungal activity of different drugs against Aspergillus fumigatus infection

Ming Sui¹², Rongqing Zhou¹, Wenxi Yue², Mingying Wei², Keyong Zhu², Xiaoping Xie³, Guoying Li¹

¹Key Laboratory of Leather Chemistry and Engineering, Ministry of Education and College of Light Industry, Textile and Food Engineering, Sichuan University, Chengdu, Sichuan Province, China; ²Department of Wine and Food Engineering, Sichuan Technology and Business College, Dujiangyan, Sichuan Province, China; ³Department of Pediatrics, Dujiangyan People’s Hospital, Dujiangyan, Sichuan Province, China

Received April 4, 2018; Accepted May 30, 2018; Epub September 15, 2018; Published September 30, 2018

Abstract: Objective: The aim of this research was to study the anti-fungal activity of different drugs against infection by Aspergillus fumigatus. Methods: A total of 56 strains of Aspergillus fumigatus, stored and isolated from sterile specimens of patients with invasive aspergillosis, from February 2013 to February 2015, in Dujiangyan People’s Hospital, were collected. Minimum inhibitory concentrations (MIC) of amphotericin B and itraconazole against Aspergillus fumigatus were observed. Results: The range of MIC of amphotericin B against Aspergillus fumigatus was 0.0600-4.0000 μg/mL while that of itraconazole against Aspergillus fumigatus was 0.0600-8.0000 μg/mL. The mean ± standard deviation of MIC of amphotericin B against Aspergillus fumigatus (0.7130±0.0020 μg/mL) was significantly lower than that of itraconazole against Aspergillus fumigatus (1.0490±0.0130 μg/mL; P<0.05). There was no difference in 50% MIC of the two drugs but 90% MIC of amphotericin B was increased compared to that of itraconazole. Conclusion: The anti-fungal activity of amphotericin B against Aspergillus fumigatus was better than itraconazole. Amphotericin B is, therefore, recommended for clinical use.

Keywords: Aspergillus fumigatus, amphotericin B, itraconazole, invasive aspergillosis, minimum inhibitory concentrations

Introduction

Invasive aspergillosis (IA) is commonly detected in immunocompromised individuals. Its incidence rate is increased with widespread promotion of bone marrow transplantation, enhanced incidence rate of diabetes, and increased use of drugs such as glucocorticoids, broad-spectrum antibiotics, and immunosuppressive agents [1-3]. It has been reported that IA often occurs in lung infections. Aspergillus fumigatus is the most common conditional pathogen in patients with IA of the lungs, accounting for approximately 40% of all patients diagnosed with pulmonary fungal infections [4]. IA patients have low specificity in symptoms. It has been reported that about 30% of IA patients are undiagnosed [5]. In addition, the mortality rate of IA patients has remained as high as 50%, although anti-fungal drugs have achieved rapid development in recent years. IA is the main reason for increasing mortality rates of immunocompromised patients [6]. Therefore, using currently available anti-fungal drugs to improve therapeutic effects in IA patients is an important issue.

Recently, it has been reported that resistance of Aspergillus to azole antibiotics has reached drug-resistant range, with in vitro drug sensitivity testing of the fungus an important means of solving the problem [7]. However, in vitro drug sensitivity testing of Aspergillus is not included in routine examinations in many hospitals [8, 9]. This present study detected the sensitivity of 56 collected strains of Aspergillus fumigatus to azole and amphotericin using Etest assay and explored in vitro drug sensitivity of Aspergillus fumigatus, providing a data reference for treatment of anti-Aspergillus fumigatus in clinical practice [9].

Materials and methods

Objects of study

A total of 56 strains of Aspergillus fumigatus, isolated and stored in Dujiangyan People’s Hos-
hospital, from February 2013 to February 2015, were enrolled in this study, having been collected from different patients diagnosed with IA. All Aspergillus fumigatus strains were obtained from sterile site specimens of patients. They were isolated and cultured, repeatedly, and confirmed through molecular biology assay. Strains with contaminant microorganisms were excluded. All strains were cryopreserved in 10% glycerol at -80°C. This study was approved by the Ethics Committee of Dujiangyan People’s Hospital and specimens were collected with informed consent of the patients.

Isolation and culture of fungi

Specimens collected from sterile sites of patients were placed in a test tube containing sterile water. The tube was shaken to spread fungi into the water. A suitable amount of fungus suspension was then dipped using a one-time inoculating loop, inoculated into Sabouraud medium (Shanghai Honsun Biological Technology Co., Ltd.) using the streak plate method, and cultured in an incubator for 2-4 days. Next, the morphology of suspicious colonies was observed under a microscope. A single suspicious colony was picked up to prepare into fungus suspension. The above operations were repeated and molecular biological identification was then conducted until pure Aspergillus fumigatus was obtained [10].

Molecular biology identification of Aspergillus fumigatus

Real-time fluorescent quantitative polymerase chain reaction (PCR) was employed to amplify the internal transcribed spacer (ITS) of ribosomal deoxyribonucleic acid of the Aspergillus. Sequences of primer ITS1: 5'-TCCGTAGGTGACCCCGCGG-3’, and that of ITS4: 5'-TCTTCCGCTTATATGC-3’. β-actin primer was designed as a negative control. EasyTag PCR SuperMix kit was purchased from Beijing TransGen Biotech and GIBCO-11732020 (Beijing Donggeboye Biological Technology Co., Ltd.) was used for detection. Volume of the reaction system was 50 μL. Reaction conditions: pre-denaturation at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 1 minute, and extension at 72°C for 30 s seconds, for a total of 30 cycles. The amplified product was sent to Shanghai Sangon Biotech for ITS sequence determination and Aspergillus was identified to species level [11]. See Table 1.

Preparation of Aspergillus fumigatus spore suspension

All 56 strains of Aspergillus fumigatus were quickly dissolved, inoculated into Sabouraud medium (Shanghai Zeye Biotechnology Co., Ltd.) in an appropriate amount, and cultured at 35°C for 3 days. Next, 5 mL normal saline with 0.1% Tween 20 (Shanghai Shiji Industrial Co., Ltd.) was added into the culture dish and mixed with strains to make a suspension. This was filtered through 10 layers of sterile gauze to prepare into Aspergillus spore suspension. Concentration of suspension was adjusted to 0.5 McFarland standard, using Roswell Park Memorial Institute (RPMI) 1640 medium (Beijing ZEPING Bioscience & Technologies Co., Ltd.) at a wavelength of 590 nm, with a DENSICHECK nephelometer (BioMérieux Clinical Diagnostics (Shanghai) Co., Ltd.). It was then diluted 100 times [12].

Preparation of anti-fungal drugs with gradient concentrations

RPMI 1640 medium was prepared into RPMI 1640 medium solution containing 2% glucose and anti-fungal drugs were diluted (doubled) (Amphotericin B: 0.0324-16.0000 μg/mL, Shanghai Shfeng Biological Technology Co., Ltd., itraconazole: 0.0152-8.0000 μg/mL, Shanghai Baoman Biotechnology Co., Ltd.) with 10 gradient concentrations for each drug.

Etest in vitro drug sensitivity testing

A total of 100 μL/well amphotericin B was added into wells 1-10 of a U-bottom plate (Shanghai Hengfei Biotechnology Co., Ltd.), from low to high concentrations, and 100 μL/ well itraconazole was added into wells 11-20 of the U-bottom plate in ascending order of concentration. Next, wells 1-20 were added with 100 μL Aspergillus fumigatus spore suspension, well 21 was added with 100 μL drug-free medium + 100 μL Aspergillus fumigatus spore suspension as positive control, and well 22 was added with 100 μL drug-free medium + 100 μL spore-free saline as negative control. Afterward, Candida parapsilosis ATCC222019 was used as a quality-control strain. The experimental method was the same as above, followed by incuba-
Activity of different anti-fungal drugs against Aspergillus fumigatus infection

Observation indicators

Minimum inhibitory concentrations (MIC) of amphotericin B and itraconazole were observed. If the detected MIC result was lower than the minimum test concentration, then the minimum test concentration was deemed as the concentration. If MIC result was higher than the highest test concentration, doubling of the maximum test concentration was considered as the concentration [14].

Statistical analysis

SPSS 19.0 software (Asia Analytics Formerly SPSS China) was used for analysis. Measurement data are expressed as mean ± standard deviation (X ± sd). K-S test was adopted to analyze conformities of measurement data to normal distribution, with t-test for data meeting normal distribution and rank sum test for data not meeting normal distribution. Drug sensitivity analysis software Who-net 5.4 was applied to calculate the allowable range of MIC, 50% MIC (MIC\(_{50}\)), and 90% MIC (MIC\(_{90}\)).

Results

Features of Aspergillus fumigatus

Aspergillus fumigatus colonies were dark green/smoke green with velvet or flocculent surfaces, white edges, and colorless or yellow-brown hirtellous backs. Under the microscope, conidial heads were cylindrical and apical cysts were stumpy or flask-shaped. The sterigmata were monolayered and distributed in the upper part of the apical cysts, accounting for about four-fifths, and the conidia were spherical, rough, and barbed. Specific details are shown in Table 2.

Results of drug sensitivity testing of the quality-control strain

Candida parapsilosis ATCC22019 acted as a quality-control strain in this study. The quality-control result of in vitro resistance testing to amphotericin B was 0.5000 μg/mL, within the MIC allowable range (0.2500-1.0000 μg/mL) [14]. The result of in vitro resistance testing to azole was 0.2500 μg/mL, also in the allowable range of MIC (0.0640-0.5000 μg/mL) [15] as shown in Table 3.

Results of MIC allowable range and quality-control test of candida parapsilosis ATCC22019 (μg/mL)

<table>
<thead>
<tr>
<th></th>
<th>Amphotericin B</th>
<th>Itraconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC allowable range</td>
<td>0.2500-1.0000</td>
<td>0.0640-0.5000</td>
</tr>
<tr>
<td>Quality-control test results</td>
<td>0.5000</td>
<td>0.2500</td>
</tr>
</tbody>
</table>

Note: MIC, minimum inhibitory concentrations.

In vitro drug sensitivity test results for 56 strains of Aspergillus fumigatus

MIC against Aspergillus fumigatus ranged from 0.0060 to 4.0000 μg/mL for amphotericin B and 0.0600 to 8.0000 μg/mL for itraconazole. Mean ± standard deviation of MIC of amphotericin B against Aspergillus fumigatus was significantly smaller than that of itraconazole against Aspergillus fumigatus (0.7130±0.0020 μg/mL vs. (1.0490±0.0130) μg/mL, P<0.05). There was no difference in MIC\(_{50}\) of the two drugs but the MIC\(_{90}\) of amphotericin B was high-

Table 1. Primer sequence

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>β-actin primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS1: 5’-TCCGATGCTGACACCTGCCG-3’</td>
<td>F: 5’-TCACCCACACCAATAA-3’</td>
</tr>
<tr>
<td>ITS4: 5’-TCCCTCGTATTGATATGC-3’</td>
<td>R: 5’-CTCATCACACACCAATAA-3’</td>
</tr>
</tbody>
</table>

Note: ITS, internal transcribed spacer.

Table 2. Characteristics of Aspergillus fumigatus colonies

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Characteristic conforms to the number of strains (n, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color of bacterial colony</td>
<td>Dark green: 38 (67.86), Smoke green: 18 (32.14)</td>
</tr>
<tr>
<td>Surface of the colony</td>
<td>Velvet: 29 (51.79), Flocculent: 27 (48.21), White edge of colony: 52 (92.86)</td>
</tr>
<tr>
<td>Back color of the colony</td>
<td>Colorless: 41 (73.21), Colorless or yellow-brown: 15 (26.79), Hirtellous back: 56 (100.00)</td>
</tr>
</tbody>
</table>

Table 3. Results of MIC allowable range and quality-control test of candida parapsilosis ATCC22019 (μg/mL)

<table>
<thead>
<tr>
<th></th>
<th>Amphotericin B</th>
<th>Itraconazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC allowable range</td>
<td>0.2500-1.0000</td>
<td>0.0640-0.5000</td>
</tr>
<tr>
<td>Quality-control test results</td>
<td>0.5000</td>
<td>0.2500</td>
</tr>
</tbody>
</table>

Note: MIC, minimum inhibitory concentrations.
Activity of different anti-fungal drugs against Aspergillus fumigatus infection

Table 4. *In vitro* drug sensitivity test results for 56 strains of Aspergillus fumigatus (μg/mL)

<table>
<thead>
<tr>
<th></th>
<th>Amphotericin B</th>
<th>Itraconazole</th>
<th>Statistic</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC allowable range</td>
<td>0.0600-4.0000</td>
<td>0.0600-8.0000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>1.0000</td>
<td>1.0000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIC&lt;sub&gt;90&lt;/sub&gt;</td>
<td>2.0000</td>
<td>1.5000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIC (X ± sd)</td>
<td>0.7130±0.0020</td>
<td>1.0490±0.0130</td>
<td>2.249</td>
<td>0.027</td>
</tr>
</tbody>
</table>

Note: MIC, minimum inhibitory concentrations; MIC<sub>50</sub>, 50% MIC; MIC<sub>90</sub>, 90% MIC.

Discussion

Aspergillus fumigatus is a conditional pathogen widely found in nature. Its resistance to drugs has become increasingly high, with the widespread use of anti-fungal drugs [15, 16]. One study has shown that the resistance rate of Aspergillus fumigatus to itraconazole is approximately 40% [17]. Forty percent is a figure worthy of vigilance, as there are fewer types of anti-fungal drugs than antibacterial drugs. In this study, resistance of Aspergillus fumigatus to amphotericin B and itraconazole was examined, aiming to provide guidance for clinical treatment.

In this study, 56 strains of Aspergillus fumigatus, identified through molecular biology in Dujiangyan People’s Hospital, were collected.

Results of this study indicated that the MIC range of amphotericin B against Aspergillus fumigatus was 0.0600-4.0000 μg/mL while that of itraconazole was 0.0600-8.0000 μg/mL. Resistance of Aspergillus fumigatus to amphotericin B was superior that of itraconazole. The mean ± standard deviation of MIC of amphotericin B was also overtly lower than that of itraconazole, showing a statistically significant difference. Itraconazole acts on cell membranes of Aspergillus fumigatus by inhibiting 14-alpha-demethylase and impeding synthesis of Aspergillus fumigatus cell membranes. In this way, itraconazole achieves its purpose of inhibiting the growth of Aspergillus fumigatus [20]. Amphotericin B also acts on cell membranes. It can bind to the sterols and increase the permeability of cell membranes, causing massive loss of components and exerting anti-fungal activity [21]. Both drugs act on cell membranes but the resistance mechanism of Aspergillus fumigatus to itraconazole does not interfere with the anti-fungal action of amphotericin B. Seyedmousavi et al. reported that the resistance mechanism of Aspergillus fumigatus to itraconazole does not interfere with the anti-fungal action of amphotericin B [22]. Moreover, Seyedmousavi et al. reported, in 2017, that amphotericin B had an excellent therapeutic effect in patients that were resistant to azoles due to immunosuppression caused by neutrophil granulocytopenia and steroid treatments [23]. Therefore, amphotericin B exerts a good therapeutic effect in patients with aspergillosis due to Aspergillus fumigatus. An international expert group meeting in 2015 also included amphotericin B in first-line treatment drugs for aspergillosis [24]. However, amphotericin B still has side effects.
It changes not only the permeability of fungal cell membranes but also the permeability of patient cell membranes, causing hypokalemia due to potassium leakage [25]. Therefore, application of amphotericin B must be performed in strict accordance with doctor’s advice. This study also had certain limitations. The relationship between separation sites of Aspergillus fumigatus and MIC against Aspergillus fumigatus was not examined or discussed. Future studies should have improved experimental design with further discussion carried out.

In conclusion, amphotericin B is superior to itraconazole, in terms of activity against Aspergillus fumigatus, and is recommended for clinical use. However, investigation into the antifungal activity of anti-fungal drugs requires further evidence from massive test results of strains. In addition, the results of in vitro drug testing for this present study should be mutually verified with clinical therapeutic effects.

Disclosure of conflict of interest

None.

Address correspondence to: Guoying Li, Key Laboratory of Leather Chemistry and Engineering, Ministry of Education and College of Light Industry, Textile & Food Engineering, Sichuan University, No.133 Kehua North Road, Chengdu 610000, Sichuan Province, China. Tel: +86-028-85405836; E-mail: liguoying@163.com

References


Activity of different anti-fungal drugs against Aspergillus fumigatus infection


