Original Article

Antifungal activity of terrestrial Streptomyces rochei strain HF391 against clinical azole-resistant Aspergillus fumigatus

Hadizadeh S1, Forootanfar H2, Shahidi Bonjar GH3, Falahati Nejad M4, Karamy Robati A1, Ayatollahi Mousavi SA1*, Amirporrostami S1

1 Department of Medical Mycology & Parasitology, Faculty of Medicine, Kerman University of Medical Sciences, Kerman, Iran
2 Herbal and Traditional Medicines Research Center, Kerman University of Medical Sciences, Kerman, Iran
3 Department of Plant Pathology & Biotechnology, College of Agriculture, Bahonar University of Kerman, Iran
4 Student Research Committee, Mazandaran University of Medical Sciences, Sari, Iran

*Corresponding author: Seyed Amin Ayatollahi Mousavi, Department of Medical Mycology and Parasitology, School of Medicine, Kerman Medical University, Kerman, IR Iran. Tel: +98-3432450295; Email: aminayatollahi@kmu.ac.ir

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Abstract

Background and Purpose: Actinomycetes have been discovered as source of antifungal compounds that are currently in clinical use. Invasive aspergillosis (IA) due to Aspergillus fumigatus has been identified as individual drug-resistant Aspergillus spp. to be an emerging pathogen opportunities a global scale. This paper described the antifungal activity of one terrestrial actinomycete against the clinically isolated azole-resistant A. fumigatus.

Materials and Methods: Soil samples were collected from various locations of Kerman, Iran. Thereafter, the actinomycetes were isolated using casein-nitrate-agar medium and the most efficient actinomycetes (capable of inhibiting A. fumigatus) were screened using agar block method. In the next step, the selected actinomycete was cultivated in starch-casein-broth medium and the inhibitory activity of the obtained culture broth was evaluated using agar well diffusion method.

Results: The selected actinomycete, identified as Streptomyces rochei strain HF391, could suppress the growth of A. fumigatus isolates which was isolated from the clinical samples of patients treated with azoles. This strain showed higher inhibition zones on agar diffusion assay which was more than 15 mm.

Conclusion: The obtained results of the present study introduced Streptomyces rochei strain HF391 as terrestrial actinomycete that can inhibit the growth of clinically isolated A. fumigatus.

Keywords: Actinomycetes, Antifungal Agents, Aspergillus fumigatus, Azoles resistant

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Introduction

Among the human pathogenic species of Aspergillus, A. fumigatus is perhaps the most devastating of Aspergillus-related diseases, followed by A. flavus, A. terreus, A. niger, and the model organism, A. nidulans [1-2]. Aspergillus fumigatus is a ubiquitous saprophytic mold that forms airborne spores (conidia). Humans inhale, on average, hundreds of these infectious propagules daily [3]. A. fumigatus pathogenesis and progression are the result of both fungal growth and the host response [4]. Invasive aspergillosis (IA) can cause a wide range of human ailments depending on host immune function [5]. Pathogenesis and virulence of aspergillus occurs when the host response is either too strong or too weak [6]. The types of hosts that are susceptible to invasive aspergillosis are the leukemic patients; hematopoietic stem cell transplant recipients, leukemia; patients on prolonged corticosteroid therapy, which is commonly utilized for the prevention and/or treatment of graft-versus-host disease in transplant patients; individuals with genetic immunodeficiencies such as chronic granulomatous disease (CGD); and individuals infected with human immunodeficiency virus [7]. In these patients, resistance is most commonly observed in A. fumigatus, and the isolates may be resistant to only itraconazole (ITZ) or exhibit a multi-azole or panazole-resistant phenotype. The phenotype depends on the underlying resistance mechanism, which commonly involves point mutations in the cyp51A-gene, the target for antifungal azoles [8].
In recent years the microorganisms have become important in the study of novel active compounds, secondary metabolites and chemical structure exhibiting antimicrobial may serve as model system in the discovery of new drugs [9]. The use of chemical fungicides has led to deteriorating human health and development of pathogen resistance to fungicide. Actinomycetes are the main source of antifungal. The antagonistic activity of actinomycetes is used for the bio-control of fungal diseases [10]. Actinomycetes produce about 75% of commercially and medically useful antibiotics [11-12]. Thus, the search for new antibiotics from these bacteria has gained importance. For example, it had been discovered in Egypt that a strain of Streptomyces spp., produced a strong antifungal antibiotics [13]. Furthermore, a research in Turkey for an antibacterial agent, producing Streptomyces spp. [14] and in China, a new strain of Streptomyces was discovered that kills certain pathogenic fungi [15].

Among the different types of drugs, secondary metabolites of actinomycetes including antibiotics with diverse chemical structure and biological activities have occupied a prominent position in the pharmaceutical industry [16]. This study was explored for the isolation characterization of native actinomyces for antifungal metabolites, to screen a new antifungal compound against drug resistant A. fumigatus.

**Material and Methods**

**Fungal strains**

In the current study, an azole-resistant strain (IFRC 500, Invasive fungi Research Center, Mazandaran University of Medical Sciences), previously isolated from Bronchoalveolar Lavage (BAL) and identified by molecular methods were used. The resistant strain harbored an L98H amino acid substitution and a 34-bp tandem repeat in the cyp51A gene promoter region, and exhibited an itraconazole minimum inhibitory concentration (MIC) of > 16 μg/ml. Stock cultures for the transient working collections were cultured on malt extract agar (MEA, Difco, Beckton, Dickinson, and Company, Franklin Lakes, NJ, USA) at 35°C for 48 h until use.

**Collection of soil samples**

100 soil samples were collected from different points of Kerman City, Iran. The samples were taken up to a depth of 20 cm after removing approximately 3 cm of the soil surface and the samples were placed in polyethylene bags to avoid external contamination and kept in 4°C until pretreatment.

**Isolation of actinomycetes**

For the isolation of actinomycetes, various methods were performed on the basis of different sources and media [17]. Soil samples were processed by serial dilution method and cultured by spread plate technique on starch-casein-agar (SCB) and incubated at 37°C for 2 weeks. Slants containing pure cultures were stored at 4°C until further examination [18].

**Identification of active actinomycetes**

Various levels for the identification of actinomycetes were used such as: i) Chemo-taxonomical level: identified based on chemical variation and characters in all genera of actinomycetes. ii) Classical level: identified based on macroscopic and microscopic methods and other properties such as the color of colonies culture. iii) Molecular level: the 16S rRNA partial gene sequences obtained from active isolate compared with other bacterial sequences by using PubMed - NCBI BLAST search [17].

**Screening of the antifungal activity**

**Spread-plate method**

The antifungal activity of actinomycetes was tested by agar plug method [19]. For the actinomycetes grown on surface of SCB medium Petri dishes, agar discs were cut out and transferred to the surface of PDA plates seeded with azole-resistant A. fumigatus. The petri dishes were incubated at 25°C to allow the growth of test organisms.

**Well Diffusion Method**

The isolated strains were transferred into the CG (Casein-Glycerin) medium in a 250 ml flask and incubated at 25°C for 15 days. Wells were made in the center of PDA plates seeded with Azole-resistant A. fumigatus. 100 μl of
the test samples were transferred into the wells and plates were incubated at 25°C. The plates were then observed for zone of inhibition.

**Assay for antifungal activity by minimum inhibitory concentration (MIC)**

MIC was determined by the antimicrobial concentrations which were prepared as 1.25, 2.5, 5, 10, 20, 40 and 80 mg/ml in DMSO: MeOH (1:1, v/v) and tested in well-method technique against the pathogen. The lowest concentration which indicated growth inhibition was selected as MIC [18].

**Results**

**Identification of Azole-resistant A. fumigatus**

Of the 50 Aspergillus isolates, 40 (80%) were *Aspergillus fumigatus*, of which one A. drugs by agar well diffusion method (CLSI M38-A2) (Figure 1).

**Table 1. Identification of strains by chemotaxonomical level**

| Chemicals         | Active strain |
|-------------------|---------------|
| Citrate           | +             |
| SIM               | +             |
| MR                | +             |
| VP                | +             |
| lactase test      | +             |
| Proteases test    | +             |
| ketones test      | -             |
Figure 2. Zone of inhibition of Actinomyces spp. kp137826 (mm) against A.fumigatus

Discussion

In our study, among fifty BAL samples, only one azoles (Clotrimazole, Itraconazole and Ketoconazole) -resistant A. fumigatus was found. In another study investigated the prevalence of azole-resistant Aspergillus spp. Only 4 azole-resistant isolates were found, which corresponds with a prevalence of 1.9% [20]. Another study showed the prevalence of 12.8% among A. fumigatus isolates that had been sent to hospitals in the Netherlands [21]. For patients with aspergillosis affected by azoles resistance A. fumigatus treated with voriconazole, the proportion of death was 48% [22]. Another study investigated the prevalence of azole-resistant Aspergillus spp, described the emergence of acquired resistance of A. fumigatus to azole compounds [23].

In our research, among the 100 actinomycete isolates, Actinomyces spp. kp137826, exhibited strong antifungal activity against azole-resistant A. fumigatus. The rate of antifungal metabolite production correlated with the growth rate of the Actinomyces spp. kp137826. Among the bacteria, actinomycetes are the important source of bioactive compounds and many clinically relevant antibiotics in use today and may continue to be so. The other study performed on 153 isolates showed broad spectrum antifungal activity [24]. Augustine reported that out of 335 isolates, 230 (69 %) isolates were active against bacteria, fungi and yeast [25]. Of the 312 Actinomycete strains from different regions, of which, 22% exhibited antifungal activity against fungi [26]. Michael et al. and Gomeset al. isolated chitinolytic actinomycetes and found its antifungal activity [27-28].

Our study shows that only Actinomyces spp. kp137826 exhibited antifungal activity against azoles-resistance A. fumigatus. The MIC of the antifungal compound was determined as 80 mg/ml and showed the highest zone of inhibition A. fumigatus (35 mm). The other study used also different concentrations e.g. 2, 4, 6, and 10% of extract were used to check antifungal activity and the minimum inhibitory concentration [29].

Streptomyces spp and Nocardia spp. also showed anti-Aspergillus activity because of observed in Netherlands in 1999 [30]. Screened287 isolates from various habitats and recorded 166, 164, 134, and 132 actinomycete isolates active against C. albicans, A. niger, M. gypseum and T. rubrum, respectively [33]. In another research, among 316 actinomycetes, 19, 67, 42, 37, 18 and 25 isolates showed activity against C. albicans, T. rubrum, M. canis, M. gyseum, A. flavus, A. fumigatus, respectively [34]. Streptomycyes rochei AK 39 also exhibited antifungal activity against dermatophytes when grown on starch-casein agar (SCA) medium with pH 7 and 37°C [35]. Several 32 [36]. In our study, we observed the antifungal activity of Actinomyces spp. actinomycetes were reported to possess anti-Aspergillus activity, e.g. Streptomycyes spp. PM- kp137826 against...
Aspergillus fumigatus, enabling the discovery of new antibiotics and hence, merit future studies.

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Authors’ contributions

S.H. in charge of data collection, sampling, doing biochemical tests and help to write the manuscript, H.F. presented the basic theme of the biochemical articles and help to do the most of the lab tests, G.H.S. help to collect the actinomycetes and their recognition and edition of manuscript, M.F. presented the method of collecting the Aspergillus resistance to Azoles and testing them all, A. KR.: help to collect the articles and write the manuscript and SA. AM. presented the basic theme of the article, writing the paper and supervised the study.

Conflicts of interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Financial Disclosure

No financial interests related to the material of this manuscript have been declared.

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