In vitro activity of nine antifungal agents against clinical isolates of Aspergillus calidoustus

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This study analyzes Aspergillus isolates from the Spanish National Centre for Microbiology collection, which were identified morphologically as members of Aspergillus section Usti. Strains were identified through the analysis of the Internal Transcribed Spacer regions and partial tubulin gene sequences. One Aspergillus pseudodefl ectus isolate and eight Aspergillus calidoustus isolates were detected in this panel of clinical strains. Terbinafine and the echinocandins micafungin and anidulafungin, were the drugs most active against these species.

Keywords: Aspergillus calidoustus, antifungal susceptibility profile, beta tubulin

Introduction

Aspergillus section Usti has recently been reviewed on the basis of chemical, molecular and morphological data [1,2]. Aspergillus insuetus, which had been synonymized with Aspergillus ustus [3], has been revived and is currently a valid species. In addition, two new species have been described in the section Usti, i.e., Aspergillus keveii and Aspergillus calidoustus. Presently, Aspergillus section Usti includes the following species: Aspergillus ustus, Aspergillus puniceus, Aspergillus granulosus, Aspergillus pseudodefl ectus, Aspergillus calidoustus, Aspergillus insuetus, Aspergillus keveii and Emericella heterothallica.

During recent years there have been several reports on the clinical importance of A. ustus. Twenty-four cases have been reported in the literature since 1971, half of them in the last three years [4–7]. Infections due to A. ustus have been associated with high mortality rates, and examination of its in vitro antifungal susceptibility profile reveals high MIC (minimum inhibitory concentration) values for many drugs [1–10]. Recently, Houbraken et al. [1,2] proposed in their taxonomic review of this section that several strains, previously identified as A. ustus, should be renamed as A. calidoustus.

The internal transcribed spacers (ITS) of the rDNA and a part of the tubulin gene (BenA) were sequenced for all Aspergillus isolates from our collection which were morphologically identified as a member of section Usti. In addition, in vitro antifungal susceptibility testing was performed by means of EUCAST mold methodology [15] to assess their susceptibility profiles.

Material and methods

Strains

Nine strains were analyzed in this investigation, all held at the Mould Collection of the Spanish National Centre for Microbiology (CNM-CM) and previously identified by morphological means as Aspergillus ustus. All were obtained from clinical samples, i.e., seven from respiratory sites, one from a vascular biopsy of an aneurysm and one from cerebrospinal fluid. Each isolate was recovered from a different patient and sent to the Spanish Mycology Reference Laboratory for identification and susceptibility testing. Strains were labelled as CNM-CM (Spanish National Centre for Microbiology-moulds culture collection), together with a unique identification number. Three strains were acquired from the culture collection of the CBS-Fungal Biodiversity Centre, i.e., two A. ustus strains,
CNM-CM4036 (CBS 113233) of unknown origin and CNM-CM4037 (CBS 239.90) from a brain abscess and one A. pseudodefectus strain CNM-CM5302 (CBS 756.74) of environmental origin.

**Morphological identification**

The strains were subcultured on different agar media to ascertain their macroscopic and microscopic morphology. The media included malt extract agar (MEA, 2% malt extract, Oxoid S.A., Madrid, Spain) and Czapek-Dox Agar (Soria Melgizo S.A., Madrid, Spain, Difco 0339-01-1). Cultures were incubated at 30°C and 37°C.

**PCR and DNA sequencing of ITS region**

Moulds were cultured in GYEP medium (0.3% yeast extract, 1% peptone, Difco, Soria Melguizo S.A) with 2% glucose (Sigma-Aldrich Quimica, Madrid, Spain), for 24–48 h at 30°C. Genomic DNA was isolated using an extraction procedure previously described [11].

DNA segments comprising the ITS1 and ITS2 regions were amplified with primer set ITS1 (5´-TCCGTAGGT GAACCTGCGG-3´) and ITS4 (5´-TCCTCCGCTTATT GATATGC-3´) [12]. Partial sequence of the β-tubulin gene was amplified using primer set βtub3 (5´-TTCCCTCCGAGACCGGT-3´) and βtub2 (5´-AGCGTGCCGGAATG-3´) [13]. All primers were synthesized by Sigma Genosys (Madrid, Spain). The reaction mixtures for ITS amplification contained 0.5 μM of each primer; 0.2 mM of each deoxynucleoside triphosphate (Applied Biosystems, Madrid, Spain); 5 μl of PCR 10 × buffer (Applied Biosystems); 2.5 U Taq DNA polymerase (Applied Biosystems); and 25 ng of genomic DNA in a final volume of 50 μl. For the portion of β tubulin, PCRs were run with a volume of 50 μl containing 1 μM of each primer; 250 μM of each deoxynucleoside triphosphate (Applied Biosystems); 1 × PCR buffer (Applied Biosystems); 2 mM MgCl₂ (Applied Biosystems); 2.5 U of Taq DNA polymerase (Applied Biosystems); and 25 ng of genomic DNA. Amplification was performed in a thermal cycler (GeneAmp PCR system 9700; Applied Biosystems) using the following cycling parameters: for ITS one initial cycle of 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 45 s at 56°C, and 2 min at 72°C, with one final cycle of 5 min at 72°C; for β tubulin 1 cycle of 5 min at 94°C and then 30 cycles of 30 s at 94°C, 45 s at 55°C, and 1–2 min at 72°C, followed by 1 final cycle of 10 min at 72°C. The PCR products were analyzed by electrophoresis on 0.8% agarose gels and visualized by transillumination (Gel Doc 2000; Bio-Rad Laboratories, Madrid, Spain) after staining with ethidium bromide (Sigma-Aldrich Quimica).

The sequencing reactions were undertaken as described previously [14], with 2 μl of reaction mixture from a sequencing kit (BigDye terminator cycle sequencing kit, Ready Reaction mixture; Applied Biosystems), 1 μM of the primers, and 3 μl of the PCR product in a final volume of 10 μl. The primers used for sequencing were ITS1 and ITS4 for the ITS region and tub1 (AATGGTGT CGCTTTCTGG) and βtub4 (5´-AGCGTGCCATGGTAC CAGG-3´) [13] for the β-tubulin gene. Sequences were assembled and edited using the SeqMan II and EditSeq software packages (Lasergene: DNASTar, Inc., Madison, Wisconsin, USA).

**Identification and phylogenetic analysis**

Identifications were based on comparisons of the sequences with ITS and β tubulin sequences of Aspergillus section Usti strains obtained from the GenBank database. The phylogenetic analyses were conducted with InfoQuest FP software, version 4.50 (BIORAD Laboratories, Madrid, Spain). The methodology used was maximum parsimony clustering. Phylogram stability was assessed by parsimony bootstrapping with 2000 simulations. Aspergillus fumigatus CNM-CM237 was used as an outgroup to root the phylograms.

**GenBank accession numbers**

Following are the GenBank accession numbers for β tubulin fragment sequence from the strains included in this work: CBS121601 FJ624456; CNM-CM2105 FJ624457; CNM-CM2272 FJ624458; CNM-CM237 FJ624459; CNM-CM2475 FJ624460; CNM-CM3788 FJ624461; CNM-CM3927 FJ624462; CNM-CM4036 FJ624463; CNM-CM4037 FJ624464; CNM-CM4115 FJ624465; CNM-CM4212 FJ624466; CNM-CM5070 FJ624467

**Antifungal susceptibility testing**

Microdilution testing was performed following the EUCAST standard methodology [15–18]. Aspergillus fumigatus ATCC 2004305 and Aspergillus flavus ATCC 2004304 were used as quality control strains [19].

The following antifungal agents were tested: Amphotericin B (Sigma-Aldrich Quimica), itraconazole (Janssen Pharmaceutica, Madrid, Spain); voriconazole (Pfizer S.A., Madrid, Spain); ravuconazole (Bristol-Myers Squibb, Princeton, USA); posaconazole (Schering-Plough Research Institute, Kenilworth, NJ); terbinafine (Novartis, Basel, Switzerland); caspofungin (Merck & Co., Inc., Rahway, NJ); micafungin (Astellas pharma Inc., Tokio, Japan) and anidulafungin (Pfizer S.A.). The final concentrations tested range from 16 to 0.03 mg/l for amphotericin B, terbinafine, caspofungin and anidulafungin, and

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from 8 to 0.015 mg/l for itraconazole, voriconazole, ravuconazole and posaconazole. The plates were incubated at 35°C for 48 h in a humid atmosphere. Visual readings were performed with the help of a mirror. The endpoint for amphotericin B, itraconazole, voriconazole, ravuconazole, posaconazole and terbinafine was defined as the antifungal concentration that produced a complete inhibition of visual growth at 48 h. For the echinocandins the endpoint was the antifungal concentration that produced a visible change in the morphology of the hyphae as compared with the growth in control well (minimum effective concentration, MEC) [20,21]. The antifungal susceptibility testing was repeated at least three times on different days for each isolate.

Results

Identification of strains

Morphological examination showed that all nine isolates were members of *Aspergillus* section *Usti*. All grew at 30 and 37°C in contrast with the *A. ustus* strains from the CBS culture collection, which were not able to grow at 37°C.

The identification to species level was assessed by molecular methods, analyzing the ITS regions (data not shown) and partial β-tubulin gene sequences (Fig. 1). Eight out of nine strains were identified as *A. calidoustus* and one as *A. pseudodefl ectus* (CNM-CM5070). No other species were found in this collection of clinical isolates. The tubulin sequence was the only characteristic that differed between *A. calidoustus* and *A. pseudodefl ectus*. Both species shared the same ITS sequence and are able to grow at 37°C.

Antifungal susceptibility testing

The geometric mean (GM) and range of the MICs of antifungal agents for each isolate is shown in Table 1. Due to the limited number of isolates, a susceptibility pattern could not be obtained. In any case, anidulafungin and micafungin have excellent activity *in vitro* against all isolates shown in Table 1. Amphotericin B also shows significant activity *in vitro* against the clinical isolates, with the exception of one *A. ustus* and one *A. calidoustus* isolate. Finally, only one *A. pseudodefl ectus* isolate showed high MICs against terbinafine.

![Fig. 1 Phylogenetic tree of the subset of isolates included in the study obtained by using maximum parsimony phylogenetic analyses and 2000 bootstrap simulations based on a portion of the tubulin sequences. Aspergillus fumigatus CNM-CM237 was used as an outgroup to root the tree.](image-url)
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Table 1  Geometric mean (GM) and range of MICs (mg/l) of antifungal agents tested in the study.
Activity of azole drugs was very limited against A. calidoustus and A. pseudodefl ectus isolates. However, voriconazole and ravuconazole showed good activity against both A. ustus isolates.

Discussion

Non-Aspergillus fumigatus Aspergillus species are increasingly isolated from human samples. An interim analysis of the TransNet surveillance program showed that A. fumigatus caused 56% of cases of invasive aspergillosis after hematopoietic stem cell transplantation [23]. Gomez-Lopez et al. [24] reported a prevalence of 45.4% of this species in a study with 338 clinical samples.

Houbraken et al. [1] and Varga et al. [2] have recently reviewed the taxonomy of Aspergillus section Usti, describing new species based on a combination of morphology, physiology, extrolite patterns and molecular techniques. Aspergillus calidoustus has been described as the species most commonly associated with human beings. This species forms a well-defined clade with A. pseudodefl ectus in their ITS sequence but could be differentiated from this specie by analyzing a portion of the tubulin gene. Both species are able to grow at 37°C.

In recent years infections caused by A. ustus have increased significantly. This species has been reported as the agent of a number of different infections such as endocarditis, pneumonia, primary cutaneous infections, invasive pulmonary aspergillosis and endophthalmitis. Varga et al. [2] included A. ustus strains used in a previously reported paper by Panakal et al. [4] in their study, and found that these strains could be identified as A. calidoustus. This fact concurred with our results, where no A. ustus was isolated from clinical samples, and therefore allows us to assume that most of the clinical cases of A. ustus reported are due to A. calidoustus. In addition, one A. pseudodefl ectus isolate was present among the analyzed strains. This species is rarely encountered but the limited reports of its occurrence could be due to the fact of it having been misidentified in the literature as A. ustus.

MICs obtained from different groups showed low activities in vitro for most of the drugs tested [4,5,10]. Since 1974, 24 infections caused by A. ustus have been described in the literature. Amphotericin B, caspofungin, voriconazole, itraconazole and combination therapies have been used to treat these infections with a low rate response. Eight therapy successes have been described [4,5,25–27], but only four patients survived.

This study reports the susceptibility data of nine Spanish clinical isolates and three CBS strains, identified by morphological and molecular methods. Despite the fact that the number of isolates is limited, practical recommendations can be obtained from the data. It seems that A. calidoustus is the most frequent species of section Usti associated with humans. At a time when prophylaxis with azole drugs is standard practice, for certain populations of patients these resistant species could be selected, as azole drugs are not effective against them.

Susceptibility testing in vitro showed that terbinafine and the echinocandins, micafungin and anidulafungin were the most active drugs against A. calidoustus, A. ustus and A. pseudodefl ectus. Other authors have reported similar results for terbinafine [4,10]. However, no data has been found so far on in vitro activity of micafungin and anidulafungin. These two new echinocandins can be useful for the treatment of these fungi, as the in vitro susceptibility testing has shown. In addition, this study as well as another report [4], have shown effective action of amphotericin B against this group of fungi (Table 1). In summary, identification at species level for Aspergillus species has become an important issue since species with high in vitro MICs have been increasingly involved in fungal infections. A. calidoustus is a recently described, multi-resistant fungus that can be found in clinical samples, and can be accurately identified at species level by sequencing a part of the β tubulin gene. Optimal treatment for these infections has not been established yet. Susceptibility testing should therefore be performed in order to provide alternatives for the treatment of this infection.

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Other authors: no conflicts

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