Diagnostic Microbiology and Infectious Disease 89 (2017) 282–287

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Mycology

Scedosporium boydii CatA1 and SODC recombinant proteins, new tools for serodiagnosis of *Scedosporium* infection of patients with cystic fibrosis☆

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ARTICLE INFO

Article history:
Received 4 June 2017
Received in revised form 18 August 2017
Accepted 21 August 2017
Available online 26 August 2017

Keywords:
Scedosporium species
Cystic fibrosis
Serodiagnosis
Catalase
Cu,Zn superoxide dismutase, recombinant proteins

ABSTRACT

Scedosporium species rank the second among the filamentous fungi colonizing the airways of patients with cystic fibrosis (CF), after *Aspergillus fumigatus*. In CF, these fungi may cause various respiratory infections similar to those caused by *A. fumigatus*, including bronchitis and allergic broncho-pulmonary mycoses. Diagnosis of these infections relies on the detection of serum antibodies using crude antigenic extracts. However, many components of these extracts are common to *Scedosporium* and *Aspergillus* species, leading to cross-reactions. Here, 5 recombinant proteins from *S. apiospermum* or *S. boydii* were produced, and their value in serodiagnosis of *Scedosporium* infections was investigated by enzyme-linked immunosorbent assay. Two of them, corresponding to the *Scedosporium* catalase A1 or cytosolic Cu,Zn-superoxyde dismutase, allowed the detection of *Scedosporium* infection, and the differentiation with an *Aspergillus* infection. These recombinant proteins therefore may serve as a basis for the development of a standardized serological test.

1. Introduction

Although usually saprophytes commonly found in contaminated soils and polluted water (Rougeron et al., 2015), *Scedosporium* species may be responsible of a wide spectrum of infections ranging from subcutaneous mycetoma to disseminated infections in immunocompromised patients (Cortez et al., 2008; Guarro et al., 2006). The genus *Scedosporium* is composed at least of 10 species with 5 principal species: *Scedosporium apiospermum*, *Scedosporium boydii, Scedosporium aurantiacum*, *Scedosporium minutisporum* and *Scedosporium dehoogii,* are known today in the genus *Scedosporium* (Gilgado et al., 2005, 2008; Lackner et al., 2014b). Among these, *S. boydii* and *S. apiospermum* which are very close phylogenetically, are the main pathogenic species in Europe (Sedlacek et al., 2015; Ziesing et al., 2016; Zouhair et al., 2013).

In patients with cystic fibrosis (CF), the prognosis essentially depends on pulmonary lesions, which result from the frequent colonization of the airways by bacteria or filamentous fungi and the subsequent inflammatory reaction. Among filamentous fungi colonizing the CF airways, *Scedosporium* species rank the second, after *Aspergillus fumigatus* (Pihet et al., 2009; Borman et al., 2010) with a prevalence ranging from 2% to 17.4% (Blyth et al., 2010; Cimon et al., 1995, 2000; Heath et al., 2009; Manso et al., 2011; Paugam et al., 2010; Sedlacek et al., 2015; Ziesing et al., 2016). In CF, these fungi are usually responsible for a chronic colonization of the airways (Defontaine et al., 2002; Zouhair et al., 2013), which may sometimes lead to true respiratory infections, similar to those caused by *A. fumigatus* like bronchitis and allergic broncho-pulmonary mycoses (Cimon et al., 2000; Vázquez-Tsuji et al., 2006). In addition, several cases of severe disseminated infections have been reported in CF patients following lung transplantation (Castiglioni et al., 2002; Husain et al., 2005; Morio et al., 2010; Sahi et al., 2007; Symoens et al., 2006).

Very few biochemical studies have been performed so far regarding *Scedosporium* species. These studies focused on the cell wall polysaccharides (β-glucans, rhamnomannans and peptidorhamnomannans) and glycolipids (glycosylceramides) (Pinto et al., 2002). In addition, hydrolytic enzymes thought to be involved in the host tissue invasion such as the major alkaline protease Alp1 (Larcher et al., 1996), some metalloproteases (Pereira et al., 2009; Silva et al., 2011) and phosphatases (Kiffer-Moreira et al., 2007) have been identified, as well as enzymes possibly involved in evasion to the host oxidative response including a cytosolic Cu,Zn-superoxide dismutase (SOD)

☆ AM, AC, JPB and MJJF are members of the ECMM/ISHAM (European Confederation of Medical Mycology/International Society for Human and Animal Mycology) working group *Fungal respiratory infections in Cystic Fibrosis* (*Fri-CF*).

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https://doi.org/10.1016/j.diagmicrobio.2017.08.013
0732-8893/

called SODC (Lima et al., 2007) and 3 catalases (Mina et al., 2015a). Nevertheless, until recently, only 3 *Scedosporium* proteins have been identified and fully characterized, namely Alp1, SodC and the major catalase called CatA1 (Larcher et al., 1996; Lima et al., 2007; Mina et al., 2015a), and only 2 genes, encoding the last 2 proteins, were sequenced (Lima et al., 2007; Mina et al., 2015b).

The diagnosis of fungal infections is currently based on the detection of serum antibodies produced against the causative agent using crude antigenic extracts. No standardized kits are commercialized today for serodiagnosis of *Scedosporium* infections, which is performed only in specialized laboratories, and counter-immunoelectrophoresis (CIE) is currently the unique method available (Chabasse et al., 1991; Cimon et al., 1995, 2000). Nevertheless, many proteins or polysaccharides are shared by *A. fumigatus* and *Scedosporium* species, which therefore may lead to immune cross-reactions in routine serological tests and false positive serological results. Cross-reactions in serodiagnosis of *Aspergillus* infections using crude antigenic extracts have been reported with related taxa belonging to the Eurotiomycetes or even with some dematiaceous fungi like the Sordariomycete *Stachybotrys atra* (Piechura et al., 1985; Vojdani, 2004), and therefore may occur with other Sordariomycetes like *Scedosporium* species. Therefore, it may be difficult to differentiate a *Scedosporium* infection from an *Aspergillus* infection while treatment of their treatment may be really different. Indeed, whereas *A. fumigatus* is usually susceptible to voriconazole or the echinocandins, *Scedosporium* species exhibit a very low susceptibility to all current systemic antifungals (Alastruey-Izquierdo et al., 2007; Gilgado et al., 2006; Lackner et al., 2014a), leading clinicians to consider these fungi as therapy-refractory pathogens (Guarro et al., 2006) and to use double or triple combinations of antifungals in some cases (Schwarz et al., 2017).

Antibodies directed towards *A. fumigatus* dipeptidylpeptidase (DPP) V (initially considered as a chymotrypsin) or catalase A1 are well-known markers of an *Aspergillus* infection since the works of Biguet et al. (1967) and Tran Van Ky et al. (1968). In addition, in *A. fumigatus*-colonized CF patients, anti-catalase A1 antibodies were shown to be associated with a poor lung function (Schønheyder et al., 1988), and among the 8 recombinant proteins evaluated by Sarfati et al. (2006), rCatA1 and rDPPV revealed to be promising tools for serodiagnosis of *Aspergillus* infections. Comparative analysis of the catalase A1 protein sequences from *S. boydii* and *A. fumigatus* showed that there were enough differences between them to develop a sensitive and specific serological test. This was further supported by the specificity of an ELISA assay previously developed using the purified native *S. boydii* catalase A1 (Mina et al., 2015a). Whatever the *Scedosporium* species involved, sera from *Scedosporium*-infected patients were clearly differentiated from those of patients with an *Aspergillus* infection. In the present study, 5 recombinant proteins were produced derived from *S. boydii* catalase A1 or from other *Scedosporium* proteins potentially interesting for serodiagnosis, i.e. DPPV, but also SODC and Alp1 since homologous proteins in *A. fumigatus* (called Asp f 13 for Alp1) were shown to be major antigens/allergens for this fungus (Gautam et al., 2007; Hamilton et al., 1995, 1996). Using these recombinant proteins, we investigated the antibody response in several groups of CF patients with or without an *A. fumigatus* or a *Scedosporium* infection, and evaluated their value as serodiagnostic tools to discriminate *Aspergillus* and *Scedosporium* infections and to differentiate between an airway colonization by *Scedosporium* species and a respiratory infection.

2. Materials and methods

2.1. Production of S. boydii CatA1 recombinant protein in Pichia pastoris

Pichia pastoris GS115 strain and pHIL-D2 expression vector (Invitrogen, Cergy Pontoise, France) were used to produce recombinant *S. boydii Sb*CatA1 with an histidine tag, r*Sb*CatA1-$(His)_6$. A *P. pastoris* codon-optimized version of *S. boydii CATA1* coding sequence (Genbank accession number KR105769) was synthesized by Genscript (Piscataway, NJ). This codon-optimized catalase A1 was cloned into EcoRI site of the pHILD-2 plasmid. The pHIL-D2 recombinant plasmid carrying the *SbCATA1-(His)$_6$* gene was used to transform *P. pastoris* according to the manufacturer's recommendations (Invitrogen).

For protein production, a single colony was inoculated in 10 mL Yeast extract-Peptone-Dextrose broth and incubated for 2 days while shaking (150 rpm) at 30 °C. One milliliter was added to 50 mL BMY medium [Yeast extract 1%, peptone 2%, potassium phosphate 100 mM pH 6, Yeast nitrogen base 1.34%, biotine 4×10^{-5}%] supplemented with glycerol 1% and incubated for 24 h at 30 °C. After centrifugation (10 min, 3000 *g*), the pellet was washed twice with sterile water. Cells were resuspended ($A_{600} = 1.5$) in 50 mL BMY medium supplemented with methanol 0.5% and incubated for 3 additional days while shaking (150 rpm) at 30 °C with daily additions of 1 mL methanol to induce the protein production. The culture was then centrifuged (10 min, 3000×*g*) and the recombinant protein was purified from the supernatant on high affinity Ni-charged resin according the manufacturer's instructions (GenScript). Purification of r*Sb*CatA1-$(His)_6$ was checked by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and its concentration was determined using the Qubit® 2.0 Fluorometer (Invitrogen).

2.2. Production of S. boydii CatA1 and SODC and S. apiospermum Alp1 and DPPV recombinant proteins in *Escherichia coli*

Escherichia coli BL21(DE3) (Invitrogen) and the expression vector pET26b(+) (Novagen, Merck Millipore, Carlsbad, CA) were used to express *Sb*CatA1, *Sb*SODC, *Sa*Alp1 and *Sa*DPPV recombinant proteins with a glutathione-S-transferase (GST) tag. Optimized coding sequence for *GST* expression *in E. coli* was cloned into pET26b(+) between NdeI and BamHI restriction sites. *CATA1*, *SODC*, *ALP1* and *DPPV* coding sequences were synthesized as codon-optimized genes for expression in *E. coli* (Genscript). The sequences were synthesized based on the original nucleotide sequence of *S. boydii CATA1* (accession number KR105769) or *SODC* (Lima et al., 2007) gene, or on *S. apiospermum ALP1* (SAPIO_CDS3715) or *DPPV* (SAPIO_CDS5066) coding sequences. As the first amino acids of the deduced protein sequences (29 for CatA1, 21 for Alp1 and 26 for DPPV) contained a predicted signal peptide motif, the corresponding nucleotides were removed from the gene sequence. DNA fragments were cloned with *GST* tag into pET26b(+). Transformants were then confirmed by restriction enzyme digestion as containing an insert of the correct size. To express these recombinant proteins and GST (control), the constructed recombinant plasmids pET26b(+) carrying either *GST-SbCATA1*, *GST-SbSODC*, *GST-SaALP1*, *GST-SaDPPV* or the *GST* gene were transformed into *E. coli* BL21(DE3) strains.

For protein production, bacteria were grown overnight at 37 °C in 5 ml of Luria Broth with 50 μg/ml kanamycin. One milliliter of the culture was then added to 30 ml of the same medium which were incubated while shaking (250 rpm) at 37 °C to reach an absorbance value of 0.5 at 600 nm. Protein synthesis was induced with 1 mM isopropyl-β-D-thiogalactopyronoside. After 3 h of incubation while shaking (150 rpm) at 30 °C, cells were collected and resuspended in 5 ml phosphate buffered saline 0.15 M pH 7.2 (PBS) with protease inhibitors (*Complete EDTA free*, Roche, France). Cells were then sonicated on ice (3 × 30 s at a setting of 8 and 70% duty cycle; Branson Sonifier 450, Fisher Scientific, Illkirch, France), and the lysate was centrifuged (10 min, 10,000 g, 4 °C). Recombinant proteins were purified from the supernatant by affinity chromatography on immobilized glutathione according to the manufacturer's instructions (Genscript). Purification was assessed by SDS-PAGE and concentration of the recombinant proteins was determined as previously described.

2.3. Human sera

A panel of 182 serum samples from CF patients was used to evaluate the value of the obtained recombinant proteins. As described previously