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REVIEW ARTICLE

Molecular characterization of opportunistic pathogenic zygomycetes

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ABSTRACT The term zygomycosis refers to a diverse group of mycotic diseases caused by members of the orders Mucorales and Entomophthorales. These infections are frequently associated with diabetic ketoacidosis, deferoxamine treatment, cancer and its therapy, solid organ or bone marrow transplantations, extreme malnutrition and neutropenia. Although these mycoses are relatively rare, their high mortality rate underline the importance of this group of fungal infections. Molecular techniques are widely used to identify the virulence factors of clinically important fungi or to develop useful diagnostic techniques. However, application of these methods to characterize the opportunistic pathogenic nature of zygomycetes started only a few years ago. This review discusses the current state of molecular studies performed on the pathogenicity and diagnosis of zygomycetes causing opportunistic human mycosis.
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KEY WORDS

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Zygomycetes fungi are characterized by the presence of aseptate wide hyphae (coenocytic mycelia) and the formation of zygospores. They are saprophytic filamentous fungi which are ubiquitous in soil and decaying organic materials. Several species belonging in the orders Mucorales and Entomophthorales have been reported to be agents of opportunistic human mycoses, designated as zygomycosis (mucormycosis).

Within the Mucorales, *Rhizopus* seems to be most frequently involved in zygomycoses, but *Absidia*, *Rhizomucor*, *Mucor*, *Apophysomyces*, *Saksanea*, *Cunninghamella*, *Cokeromyces* and *Syncephalastrum* species have also been isolated from clinical specimens (Ribes et al. 2000; Eucker et al. 2001; Freifeld and Iwen 2004); in fact, non-*Rhizopus* species are being increasingly recognized as causative agents of opportunistic mycoses (Walsh and Groll 1999). Healthy humans are generally unaffected, but those with weakened immunity are at risk of infection. The major risk factors for the development of zygomycosis are diabetic ketoacidosis; deferoxamine treatment to manage an iron or aluminium overload; cancer and its therapy; solid organ or bone marrow transplantations; prolonged steroid use; and extreme malnutrition or neutropenia (Ribes et al. 2000; Nucci 2003; Walsh et al. 2004). The spores produced by these fungi are airborne and can be inhaled into the respiratory tract: this is the most common route for the infection of a susceptible host. Zygomycoses manifest primarily as rhinocerebral infections, but pulmonary and disseminated mycoses also occur, as do gastrointestinal and subcutaneous infections and allergic diseases. 70% of rhinocerebral infections, which account for from one-third to one-half of all zygomycoses, are associated with diabetic ketoacidosis (Ribes et al. 2000). Rhinocerebral disease has a high mortality rate, but curation is possible (depending on the state of the patient) if an early diagnosis is followed by aggressive surgical and antifungal treatment.

Conidiobolus and *Basidiobolus* species, from the order Enthomophthorales, generally cause subcutaneous and mucocutaneous infections in tropical and subtropical areas.

The diagnosis of zygomycoses is very challenging: most such infections are identified only as zygomycosis or mucormycosis, without species or at least genus determination (Ribes et al. 2000; Eucker et al. 2001; Freifeld and Iwen 2004). Currently, the definitive diagnosis of zygomycosis is achieved by biopsy and histological study of the tissue lesions (Freifeld and Iwen 2004; Walsh et al. 2004). Culturing of an isolate from a tissue sample could be of help for the species identification. However, this is often difficult, because hyphal elements may be rare in tissue specimens and they can lose their viability during the tissue homogenization prior to culturing. Whenever an isolate is successfully isolated from a clinical sample, it is very important to maintain it for further analysis. Simple strain maintenance methods are well established for the zygomycetes (Palágyi et al. 1997) and can be easily carried out in clinical laboratories. Attempts have been made to elaborate diagnostic methods based on molecular and antigen detection techniques (Jones and Kaufman 1978; Hessian and Smith 1982; Pierce et al. 1982; Yankey and Abraham 1983; Kaufman et al. 1989; Zeilander et al. 1990; Voigt et al. 1999; Wu et al. 2003), but all of them are still in the experimental phase and are not yet used in clinical practice (Ribes et al. 2000).

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Although high doses are needed for successful therapy, amphotericin B is the classical antifungal agent of choice for the treatment of zygomycosis. Currently available triazoles have proved to be inactive as sole antifungal agent against zygomycetes (Garas et al. 1999; Tawara et al. 2000; Sun et al. 2002a). An exception is posaconazole, which exhibits activity *in vitro*, in animal models and in some patients (Sun et al. 2002b; Tobon et al. 2003). Applications of drug combinations for this fungal group are weakly documented. Surgical intervention is a standard part of the treatment of localized infections.

The high mortality rate (75-95%, depending on the form of the zygomycosis) and the fact that these fungi display intrinsic resistance to the most widely used antifungal drugs underline the importance of this group of fungal infections (Ribes et al. 2000; Eucker et al. 2001).

In the past decade, advances in fungal biology have made molecular techniques essential in the study of medically important fungi. These methods are used to identify their virulence factors, to determine new therapeutic targets or to develop useful diagnostic techniques. Although molecular methods are relatively well established for the zygomycetes, and especially for the genus *Mucor*, application of these techniques to study the opportunistic pathogenic nature of these fungi started only a few years ago. We review here the current state of pathogenic and diagnostic studies on clinically important zygomycetes, based on molecular genetic approaches.

Molecular taxonomy and strain typing

Techniques used in taxonomic studies and genotyping, such as PCR with species-specific primers, hybridizations with specific probes, mitochondrial and genomic restriction fragment length polymorphism (RFLP), sequence analysis of the ribosomal DNA and/or the internal transcribed spacer (ITS) region, single-strand conformational polymorphism (SSCP), random amplified polymorphic DNA (RAPD) and pulsed field gel electrophoresis (PFGE), could serve as the basis for the development of rapid and specific DNA-based methods for the clinical diagnosis; moreover, molecular genotyping could reveal important data concerning the epidemiology of these infections.

Ribosomal DNA (rDNA) and some other gene sequences (such as actin and elongation factor 1) have been successfully used to reveal the phylogenetic relationships within the orders Mucorales (Voigt et al. 1999; O'Donnell et al. 2001; Voigt and Wöstemeyer 2001; Papp et al. 2003a) and Entomophthorales (Jensen et al. 1998). The results of these studies suggest that zygomycete systematics based primarily on morphological characteristics is highly artificial and a complete revision appears indicated (Nagy et al. 2004b). A number of markers have been identified that are potentially useful for further studies aimed at the development of diagnostic methods.

In contrast with the detailed phylogenetic studies on higher taxonomic levels, intraspecific genetic polymorphisms have been analyzed only relatively rarely in the zygomycetes. The taxonomic positions of several *Rhizomucor* strains have been determined and evaluated by ITS-RFLP (Vastag et al. 1998a; Vágvölgyi et al. 1999). On this basis, isolates were clearly demonstrated to be members of either *R. miehei* or *R. pusillus;* surprisingly, the single isolate of *R. tauricus* was identified as a mutant heterothallic *R. pusillus* strain. ITS-RFLP also distinguished *Apophysomyces elegans*, an emerging agent of zygomycosis with an increasing number of cases in India, from other clinically important zygomycetes, but the method was not able to demonstrate intraspecific polymorphism (Chakrabarti et al. 2003); microsatellite PCR fingerprinting was used to resolve this problem. ITS-RFLP and PCR fingerprinting allowed differentiation among the isolates of *Cunninghamella echinulata* and *C. bertholletiae* (Lemmer et al. 2002).

Among the varoius RFLP techniques, restriction patterns of mtDNA are frequently used in fungal taxonomy and genotyping, because this sensitive method indicates differences at a specific and even an intraspecific level. However, in the Zygomycetes, only the mtDNA organization of individual isolates of a few species have been reported, i.e. *M. racemosus* (Schramke and Orlowski 1993), *M. piriformis* (Papp et al. 1999), *R. stolonifer* (Paquin et al. 1997), *R. oryzae*, *Mortierella verticillata* and *Smittium culisetae* (Seif et al. 2005).

PFGE is also a versatile tool for molecular typing and to reveal the genetic variability at species and intraspecies levels. In the past 15 years, karyotypes of several zygomycetes, such as *Mucor circinelloides* (Vágvölgyi and Manczinger 1990; Nagy et al. 1994; Diaz-Minguez et al. 1999), *M. bainieri*, *M. mucedo*, *M. plumbeus*, *M. racemosus* (Nagy et al. 2000), *Parasitella parasitica* (Burmester and Wöstemeyer 1994) and species belonging in the genus *Micromucor* (Nagy et al. 2004) have been established. However, only a few data concerning opportunistic zygomycetes have been reported to date. The karyotypes of *Absidia glauca* strains have been revealed by rotating field gel electrophoresis (Kayser and Wöstemeyer 1991). Isolates with different mating types exhibited considerable differences in their electrophoretic karyotypes; a similar situation was observed in *M. circinelloides* (Diaz-Minguez et al. 1999).

RAPD analysis is also able to provide reproducible markers for strain identification. This method was previously used to establish specific PCR products able to differentiate between strains of several non-pathogenic zygomycete species, such as *P. parasitica* (Burmester and Wöstemeyer 1994), *M. piriformis* (Papp et al. 1997), *M. genevensis* (Vágvölgyi et al. 2001) or *Gilbertella persicaria* (Papp et al. 2001). RAPD analysis of *Rhizomucor* strains showed *R. miehei* to be genetically more homogeneous than the diverse *R. pusillus* (Vastag et al. 1999, 2000). These results strongly supported the observations of the earlier studies based on isoenzyme

analysis and carbon source assimilation assay (Vastag et al. 1997, 1998b). RAPD and the isoenzyme markers described in these works could be utilized in further studies to identify clinical and environmental isolates of *R. miehei* and *R. pusillus* and to check the accuracy of the original species identifications (Lukács et al. 2004b; Papp et al. 2004). The intraspecific variability of *Rhizopus stolonifer* and *R. oryzae* species was also examined by the RAPD method (Vágvölgyi et al. 2004a). Although only a few *R. oryzae* strains were involved in that study, the RAPD analysis appeared to support the unity of the species *R. oryzae*, which was established with the incorporation of about 30 strains originally described as independent species.

Molecular diagnostics

All types of infections caused by the zygomycetes are notorious as being difficult to diagnose and treat. Diagnosis in an early phase of the infection is essential for a successful outcome of zygomycoses. Recent attempts of diagnosis, based on the molecular and serologic techniques, are still in the experimental phase. In an early effort to apply molecular methods for diagnosis, 18S rDNA sequences with SSCP patterns were established to distinguish *Rhizopus* infections from those caused by other fungi (Walsh et al. 1995). Strains of *Rhizopus*, *Rhizomucor*, *Cunninghamella*, *Zygorhynchus* and *Mucor*, together with several clinically important non-zygomycete species, were involved in the development of a broad-range PCR assay (Van Burik et al. 1998). This method was optimized to detect fungal rDNA directly in the blood of patients, using long PCR probes. Although this non-specific assay is able to amplify the marker sequences of a broad range of fungal species, *Rhizomucor* and *Cunninghamella* proved to be non-detectable with the primers constructed in that study. The first significant advance in the development of a DNA-based identification method was the determination of 18S and 28S rDNA sequences from 42 zygomycetes involving the most common opportunistic pathogenic species (Voigt et al. 1999). Those authors designed 13 taxon-specific PCR primer pairs based on the 28S rDNA sequences to resolve the clinically most important zygomycetes, including different species of *Mucor*, *Rhizopus*, *Rhizomucor*, *Absidia*, *Cokeromyces*, *Cunninghamella*, *Basidiobolus* and *Conidiobolus*. Later, Wu et al. (2003) constructed a set of hybridization probes to detect the 18S rDNA of *Mucor* and *Rhizopus* species. Clinical strains of *Mucor*, *Rhizopus*, *Rhizomucor* and *Cokeromyces* were recently involved in an *in vitro* test of the commercially available MicroSeq D2 large subunit rDNA fungal sequencing kit (Applied Biosystems), which proved to be useful for the detection of these fungi (Hall et al. 2004).

Genetic transformation systems

The approaches most commonly used to examine the genetic background of pathogenic fungi are gene isolation, and the analysis of gene expression and gene disruption. The availability of an effective transformation system is a basic requirement for this type of molecular study.

The genetic transformation of a zygomycete fungus was first reported by van Heeswijck and Roncero (1984). They transformed a leucine auxotrophic strain of *M. circinelloides*, using a plasmid which harboured the α–isopropylmalate dehydrogenase gene (*leuA*). Their results have led to this species becoming a model organism for molecular studies on the class Zygomycetes during the past 20 years. Transformation procedures have additionally been elaborated for other zygomycetes, such as *A. glauca* (Wöstemeyer et al. 1987), *R. pusillus* (Wada et al. 1996, Yamazaki et al. 1999), *R. miehei* (Lukács et al. 2003, 2004a; Monfort et al. 2003; Vágvölgyi et al. 2004b), *Rhizopus niveus* (Yanai et al. 1990, 1991; Liou et al. 1992; Takaya et al. 1996), *R. delemar* (Horiuchi et al. 1995) and *R. oryzae* (Skory 2002, 2004; Michielse et al. 2003).

Mucor transformation protocols have traditionally been based on the $CaCl_2$/PEG-mediated methodology and necessitate protoplast formation from hyphae of young colonies or from germinating sporangiospores. The PEG-mediated transformation or electroporation of zygomycetes allows high-frequency transformations, but the introduced DNA remains almost exclusively extrachromosomal, replicating autonomously in the transformants (van Heeswijck and Roncero 1984; Revuelta and Jayaram 1986; Anaya and Roncero 1991; Iturriaga et al. 1992; Benito et al. 1995; Velayos et al. 1998; Wolf and Arnau 2002; Ács et al. 2003a; Papp et al. 2003b, 2005). In several cases, such transformants exhibit low mitotic stability. At times, integration in the genome can also be forced in these systems with the application of strong selection marker (Arnau et al. 1991; Arnau and Stroman 1993; Wada et al. 1996; Yamazaki et al. 1999) or the use of linear DNA fragments containing homologous flanking regions to drive the integration (Papp et al. 2002), but this is not the normal fate of the DNA introduced in these fungi. As a consequence, the genetic modification of the zygomycetes is generally hampered by the lack of an efficient integrative transformation system. To resolve this problem, the development of *Agrobacterium tumefaciens*-mediated transformation systems has been started for some zygomycetes, e.g. *R. oryzae* (Michielse et al. 2004), *R. miehei* (Monfort et al. 2003) and *M. circinelloides* (Nyilasi et al. 2003, 2005a), in order to achieve stable integrative transformant strains.

Most of the transformation systems involve auxotrophy complementation to select for the transformants. The most frequently used markers are the α–isopropylmalate dehydrogenase (*leuA* or *leu1*) and orotidine-5-monophosphate decarboxylase (*pyrG* or *pyr4*) genes, which complement leucine and uracil auxotrophy, respectively (van Heeswijck and Roncero 1984; Benito et al. 1995; Michielse et al. 2002; Wolf and Arnau 2002; Skory 2004). However, these selection

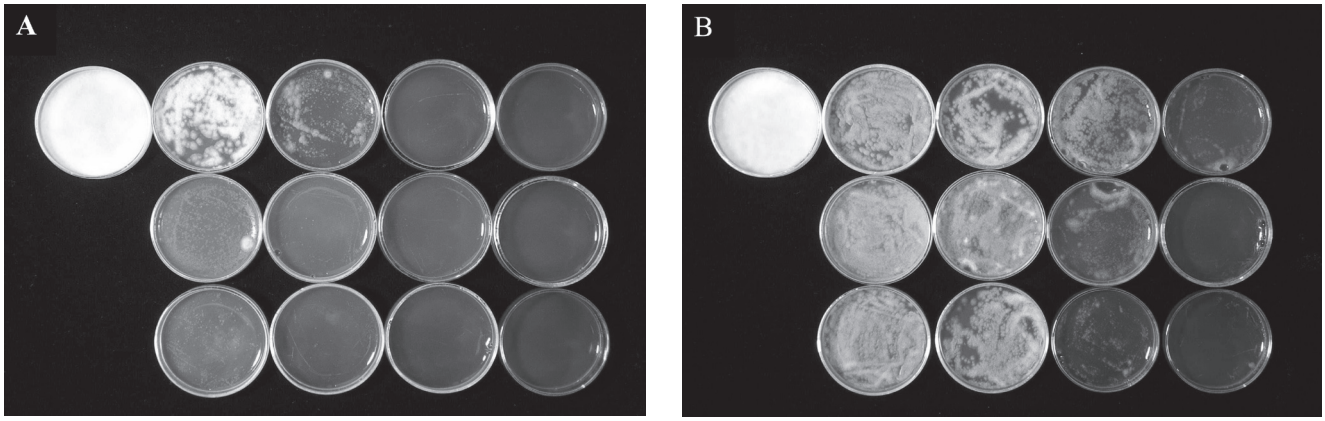

Figure 1. Effects of rose bengal and dichloran on the radial growth of *M. circinelloides*. A: colony formation of M20 on yeast extract-glucose (YEG) medium and YEG medium supplemented with 100 mg ml^{-1} hygromycin; 25, 50, 75, 100 mg ml^{-1} rose bengal (columns) and 1, 2, 3 mg ml^{-1} dichloran (rows). B: colony formation of transformant M20/A4 on YEG medium and YEG medium supplemented with 100 mg ml^{-1} hygromycin; 25, 50, 75, 100 mg ml^{-1} rose bengal and 1, 2, 3 mg ml^{-1} dichloran.

methods have the drawback that a stable auxotrophic mutant first has to be isolated from each strain that it is desired to transform. Dominant selection markers would make possible the direct transformation of wild-type strains. Unfortunately, most of the zygomycetes tested so far are highly resistant to various antibiotics. For example, *M. circinelloides* was reported to be resistant to hygromycin B, geneticin, nemoycin, oligomycin and benomyl (van Heeswijck et al. 1988). A method was recently developed for the hygromycin B-based selection of *Mucor* transformants: the sensitivity of the fungus was increased by the addition of rose bengal and dichloran to the culture medium (Fig. 1; Ács et al. 2003b; Nyilasi et al. 2005a).

To achieve heterologous gene expression in a host, it is usually necessary to combine the foreign gene with an adequate regulatory sequence of the host. Promoter sequences of the glyceraldehyde-3-phosphate dehydrogenase genes (*gpd*) have been widely used for the construction of expression vectors in different yeasts and filamentous fungi. These genes have also been isolated and characterized from a few zygomycetes: *M. circinelloides* (Ács et al. 2002; Wolf and Arnau 2002) and *R. miehei* (Ács et al. 2003c; Vastag et al. 2004). The 5' flanking region of *Mucor gpd1* has been applied as a strong and regulated promoter in an expression study (Larsen et al. 2004). With this same sequence, a dominant selection marker-based system was elaborated by Appel et al. (2004), who constructed a plasmid containing the Tn-5-derived kanamycin resistance gene combined with the promoter sequence of *Mucor gpd1*.

Identification of virulence factors

Thermotolerance, the production of efficient proteolytic, glycosidic and lipolytic extracellular enzymes (Ribes et al. 2000), siderophore production and an efficient iron transport system (Nyilasi et al. 2005b) have been suggested as the most likely virulence factors for opportunistic pathogenic zygomycetes. The isolation, cloning and disruption of a gene is an efficient means of analyzing the pathogenicity of a fungus and of determining and verifying possible virulence factors. Numerous genes encoding lipases, proteases and glycosidases have been isolated in the cases of *Mucor*, *Rhizopus*, *Rhizomucor* and other zygomycetes, but whether they play any role in the pathogenic processes remains unknown (Ribes et al. 2000). A high-affinity iron permease gene (*rFTR1*) of *R. oryzae* was recently cloned and studies to analyse its function were started by Fu et al. (2004). The transformation of the *rFTR* gene to *S. cerevisiae* partially restored the ability of an *ftr1* null mutant to grow on an iron-limited medium. Null mutant strains of *Candida albicans*, produced by gene disruption of the homologous *CaFTR* gene, previously displayed reduced virulence as compared with the wild-type strains in animal models (Ramanan et al. 2000). However, similar studies have not yet been reported with the *Rhizopus* gene. A homologous *FTR* gene of *R. miehei* and vector construction to produce null mutant strains for virulence studies were reported by Nyilasi et al. (2005c).

In the past year, sequencing of the genome of the *R. oryzae* clinical isolate RA99-880 was completed and the sequence assembly was published on the web by the *Rhizopus oryzae* Sequencing Project (Broad Institute of Harvard and MIT 2004; http://www.broad.mit.edu). An acceleration of molecular studies on the pathogenicity of the zygomycetes can be expected in consequence of this result.

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