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Studies of human pathogenic fungi: development of identification schemes, use of molecular genetic methods for their detection and evaluation of their susceptibilities

Ph.D. Thesis

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I. INTRODUCTION

1. Medically important fungi

About 200 fungal species are known to cause human infections. *Kwon-Chung* and *Bennett* (1992) provided a list of about 30 of the most important mycoses, the sources of the organisms that cause them, and the portals by which they enter the body. These diseases vary from superficial mycoses (the skin, nails, *etc.*) to deeper infections that involve the muscles, blood system and internal organs. Examples of some of the most significant mycoses found in different parts of the world include blastomycoses, coccidiomycoses, histoplasmosis, aspergillosis, cryptococcosis and candidiasis. Any of these infections can be fatal under certain conditions.

1.1. Yeasts

1.1.1. Morphology of yeasts

Yeasts are fungi that exist as single cells capable of reproducing quickly by budding or fission. As the bud is formed, it increases in size while it is still attached to the parent cell, then eventually breaks off and forms a new individual. Chains of buds, forming a short mycelium, referred to as a pseudomycelium, are sometimes produced (*e.g.* among *Candida* spp.). Fission is a simple splitting of a cell into two daughter cells by constriction and the formation of a cell wall (*Schizosaccharomyces* spp.).

Some species of fungi (*H. capsulatum*, *Blastomyces dermatitidis*, *Coccidioides immitis*, *Paracoccidioides braziliensis* and *Sporothrix schenkii*) can exist as either hyphae or single cells, and are said to be dimorphic. Many of these organisms grow as hyphae outside their hosts, but assume a yeast-like appearance inside the hosts. Various physical and chemical factors influence the conversion from hyphae to yeast cells and *vice versa*.

Some yeasts, such as most *Candida* spp., produce blastoconidia. The formation of blastoconidia involves three basic steps: bud emergence, bud growth and conidium separation. During bud emergence, the outer cell wall of the parent cell thins. Concurrently, new inner cell wall material and plasma membrane are synthetized at the site where new growth is occurring. As the bud grows, mitosis occurs, and both the developing conidium and the parent cell will contain a single nucleus. A ring of chitin forms between the developing blastoconidium and its parent yeast cell. This ring grows in to form a septum. Separation of the two cells leaves a bud scar on the parent cell wall.

On the other hand, the hyphae of some genera (*Geotrichum* spp. and *Trichosporon* spp.) routinely break up into their component cells, which then behave as spores. These spores are known as arthrospores. If the cells become enveloped in a thick wall before they separate from each other or from other hyphal cells adjoining them, they are often called chlamydospores (*C. albicans*). Traditionally, this term has been used for environmentally resistant (R) resting cells. They may be terminal or intercalary.

The morphology on an agar surface is a little similar to that of bacterial colonies. Some species produce a typical colony on a Sabouraud agar surface. *C. inconspicua* forms shallow, lacklustre white colonies with a green apple-like odour, *Saccharomyces cerevisiae* forms white to cream-coloured, peaky colonies, and *Rhodotorula mucilaginosa* forms coral red to pink colonies.

Yeast taxa may be distinguished on the basis of the presence or absence of capsules (*Cryptococcus* spp.), the size and shape of the yeast cells, the mechanism of

daughter cell formation (conidiogenesis), the formation of pseudohyphae and true hyphae, and the presence of sexual spores, in conjunction with physiological data.

1.1.2. *Candida* spp. as members of the normal human flora

Candida spp. occur most frequently as human pathogenic yeasts. There are over 100 species of *Candida*; however, some species (*C. albicans, C. tropicalis, C. parapsilosis, C. glabrata* and *C. krusei*) are isolated with widely-varying frequency from medical specimens. *C. albicans* is frequently considered part of the normal flora, especially from mucocutaneous specimen sources, unless the organism is recovered in high numbers or in pure culture. Therefore, the isolation and identification of this species from clinical specimens such as stools, urine or throat cultures may indicate colonization and not necessarily infection.

In a study investigated by *Soll et al.*, 52 healthy women (mean age 28 years ± standard deviation 11 years), who had had no episodes of oral or vaginal candidiasis during the 6-month period preceding sampling, 73% were found to carry *C. albicans*. The frequency of yeast carriage was highest in the mouth (56%), followed by the vulvovaginal (40%) and anorectal (24%) regions. *Cohen et al.* reported that the concentration of yeast and the frequency of isolation in healthy volunteers increased when the samples were obtained distally through the gastrointestinal tract. The prevalence of yeast reported as 35% in the oropharynx, 50% in the jejunum, 60% in the ileum and 70% in the colon.

While a healthy oral, gastrointestinal and vaginal mucosal surface can support a substantial, but innocuous saprophytic population of *Candida* species, the same is not true of the healthy skin. These organisms are generally not considered to comprise a major portion of the normal skin flora, being recoverable in only up to 5% of random skin cultures.

1.1.3. Pathogenicity of *Candida* spp.

The predisposing factors of opportunistic *Candida* infections include the elimination of bacterial competition following the administration of oral or parenteral antibacterial agents, the use of broad-spectrum antibiotics and significant elevations in extracellular glucose concentrations. Compromised immune systems and hormonal changes due to haematological malignancies, human immunodeficiency virus infection, drug therapy, diabetes and pregnancy may all cause the yeast to infect humans. The infection may be superficial candidiasis. Both host and environmental factors (heat, humidity, friction between skin surfaces, or frequent exposure to water) may play a role in facilitating this type of candidiasis. The same strains may also produce deep candidiasis: localized or haematogenously disseminated infections. In patients who have candidaemia, the same species may be isolated from specimens other than blood cultures at the same time (within 1 week), and colonization of different body sites may be the first sign of a systemic *Candida* infection. *Candida* spp. are emerging as important aetiological agents of nosocomial infections and infections in immunocompromised patients. However, the proportion of patients without neutropenia or severe immunosuppression who are at risk of invasive fungal infections is higher than usually thought. Carriage of the infecting strain on the hands of health-care workers may also cause nosocomial infections. *Candida* species are the fourth most frequent nosocomial bloodstream microorganisms.

On the other hand, implanted devices, such as catheters, prosthetic heart valves and joint replacements, provide pathogenic microorganisms with a surface on which they

can form an adherent biofilm. Detachment of microorganisms from the biofilm can result in septicaemia, which may respond to conventional drug therapy. Although the majority of these infections are caused by bacteria, fungal infections are becoming increasingly common. They are most often caused by pathogenic *Candida* spp., particularly *C. albicans*, which is now recognized as one of the most important agents of hospital-acquired fungal infections.

1.2. Moulds

1.2.1. Morphology of moulds

The identification of filamentous fungi depends on the microscopic and macroscopic examinations of their structures. Moulds are characterized by the development of hyphae. Three major categories of hyphae may aid in the recognition of certain groups of fungi: 1) large, aseptate or few-septate hyphae are characteristic of the Zygomycetes and more specifically of the Mucorales (*Rhizopus, Mucor, Absidia, etc.*); 2) vegetative hyphae with clamp connections indicate a fungus in the division Basidiomycota, but these hyphae are rarely seen in the medical laboratory; and 3) relatively narrow, septate hyphae are characteristic of the great majority of medically important filamentous fungi.

For all practical purposes, in the clinical laboratory filamentous fungi, which spontaneously produce their sexually reproductive structures (homothallic fungi) are limited in routine observations to a few species, *i.e. Aspergillus nidulans*, the *A. glaucus* group, *Chaetomium* and *Pseudallescheria boydii*. These organisms produce 'ascomata': perithecium and cleistothecium.

The overwhelming majority of medically important filamentous fungi are identified by colony morphology (*e.g.* colour, texture, pigmentation) and their conidiogenesis (*e.g.* solitary, in branched chains /*Cladosporium*/, sympodially, on denticles, through pores, at the apices of phialides /*Fusarium*, *Acremonium*, *Aspergillus*, *Penicillium*/, annelids, in unbranched chains /*Scopulariopsis*/, arthroconidia, chlamydospores, aleurioconidia).

1.2.2. Moulds as colonizers

Filamentous fungi may occur anywhere. The groundwater-derived public drinking water contains fungal flora, which in a German study consisted of a limited number of *Acremonium* spp., *Exophiala* spp., *Penicillium* spp. and particularly *Phialophora* spp. *Aspergillus* spp., *Fusarium* spp. and the members of Mucorales are found in the soil, practically universally in the ambient air, therefore colonizing different surfaces. The distribution of spores of different air-borne fungi can vary season by season. The inhalation of conidia by immunocompetent individuals rarely has any adverse effect, since the conidia are eliminated relatively efficiently by innate immune mechanisms.

Saprophytic filamentous fungi can be isolated from about 50% of human skin and nail samples. In a report, non-dermatophytes and dermatophytes were also found to be recoverable from the fungal flora of human toe webs in 49% and 24%, respectively. *Aspergillus*, *Fusarium*, *Alternaria*, *Cladosporium* and *Rhizoctonia* species were obtained from specimens originating from healthy and infected conjunctival sac, and *Penicillium* spp. was isolated from the contact lenses of asymptomatic contact lens wearers. Members of the

Penicillium genus are rarely reported as causative agents of infections. Moulds can also occur in human stool flora.

A. fumigatus and *S. apiospermum* are well known for their ability to colonize the respiratory tract of cystic fibrosis (CF) patients. The total colonization time varies from 10 to 56 months. The presence of *S. apiospermum* in the respiratory tract may trigger an inflammatory response that can manifest as an allergic bronchopulmonary disease.

Fungal surveillance cultures do not reliably predict systemic infection, but they are needed for the prevention of infection in high-risk patients. As a number of fungi with low virulence that were previously considered to be contaminants or harmless colonizers are emerging as pathogens associated with significant morbidity and mortality, it is now becoming necessary to know their epidemiological status and the susceptibility to commonly used antifungal agents.

1.2.3. Pathogenicity of moulds

The pathogenic behaviour of opportunistic filamentous fungi results from their specific biological features, such as the release of a high concentration of small-size conidia into the air, which are easily inhaled and able to germinate and grow at temperatures higher than 37 °C without any specific nutritional requirements. *Leenders et al.* described that species able to grow *in vitro* at 22 °C could be regarded as non-pathogenic, and species that are able to grow at 37 °C or higher could be considered potentially pathogenic. These fungi have different factors of pathogenicity, which can facilitate their tissue invasion, mainly in immunosuppressed patients. The inhaled small resting conidia enlarge and germinate, resulting in transformation into hyphae, with subsequent vascular invasion and eventual disseminated infection in the absence of an appropriate immune reaction. *A. fumigatus* produces a complement inhibitor, which arrests the opsonization and elimination of conidia by the immune system. Many *Aspergillus* species produce toxins (aflatoxin, ochratoxin A, gliotoxin, *etc.*), which may reduce the macrophage and neutrophil functions, although the role of these toxins as major virulence factors is not established. *H. capsulatum* has a gene encoding a calcium-binding protein: its presence is essential for virulence. Dermatophytes secrete proteinases, which aid the penetration.

Fungi such as *Fusarium* spp., *Curvularia* spp. and *Alternaria* spp. were previously thought to represent contamination, harmless colonization, or weak pathogens responsible for allergic forms of the disease. These infections have usually been limited to superficial mycoses, but nowadays the number of deep tissue and disseminated infections has been increasing greatly in parallel with the increasing number of immunosuppressed patients and the degree of severity of modern immunosuppressive therapies. *A. fumigatus* is responsible for the majority (85-90%) of the different clinical manifestations of severe mould infections. Accordingly, other *Aspergillus* spp. (*e.g. A. niger*, *A. flavus*, *A. terreus* and *A. nidulans*), *S. apiospermum*, *S. prolificans* and less common moulds have become important emerging pathogens which also may cause human diseases.

2. Antifungal agents and susceptibility testing methods

Fungi are eukaryotes, and consequently most agents toxic to fungi are also toxic to the host. As fungi generally grow slowly and often in multicellular forms, it is more difficult to quantify them than bacteria.

The polyene antimycotics, such as amphotericin B, nystatin and pimaricin interact with sterols in cell membranes (ergosterol in fungal and cholesterol in human cells) to form channels through the membrane, increasing the permeability, disrupting the metabolism and causing cell death. Amphotericin B is the main antifungal agent for the life-threatening fungal infections. It has broad spectrum of activity including dimorphic fungi, most of *Candida* spp., *Cryptococcus neoformans*, *Aspergillus* spp. and the Zygomycetes. Resistance to this agent is rare (some non-*albicans Candida* spp., *Fusarium* spp.). Because of its adverse effects, this drug was incorporated into liposomes, so it has significantly less nephrotoxicity. Nystatin has a broad spectrum of activity against most species of *Candida* and *Aspergillus*. *In vitro* it also inhibits the reproduction of dimorphic fungi, dermatophytes and moulds at different levels. It has a low oral bioavailability and is generally used as a topical treatment for thrush, gastrointestinal candidiasis and bronchial aspergillosis, or in the prevention of systemic fungal infections in immunocompromised patients. A lipid formulation of this antifungal agent has been developed for systemic use.

The azole antifungal agents have 5-membered organic rings containing either two (imidazoles) or three (triazoles) nitrogen atoms. These are synthetic compounds inhibiting the fungal cytochrome P_{450}-dependent enzyme lanosterol 14-α-demethylase, causing disruption of membrane (ergosterol) synthesis in the fungal cell. Ketoconazole has a range of activity including infections due to *H. capsulatum* and *B. dermatitidis*. It is also active against mucosal, cutaneous candidiasis and dermatophyte infections. Fluconazole is widely used for the prophylaxis of systemic fungal infections and the treatment of confirmed systemic *Candida* infections in immunocompromised patients. It is also effective in the treatment of cryptococcal meningitis in patients with AIDS, and in preventing relapse, when given as secondary prophylaxis. It is inactive against *Aspergillus* spp. and some species of *Candida*. It has an oral bioavailability of approximately 90%, and can be used to treat mucosal and cutaneous candidiasis too. Because it accumulates in fingernails and toenails, it can also be used to treat onychomycosis. Fluconazole is available in local, oral and parenteral formulations. Side-effects are not as common with the azoles as with amphotericin B, but life-threatening liver toxicity can arise on long-term use. Itraconazole has antifungal activity against *Candida* spp., *Aspergillus* spp. and dermatophytes. It is widely used for the treatment of fungal nail infections. It is absorbed orally and accumulates in organs, which are frequent sites for systemic fungal infections (the spleen and lungs), and it can therefore be used to treat and prevent a variety of systemic fungal infections. Voriconazole is a new extended-spectrum triazole, which is available in both oral and intravenous formulations. It has a very broad spectrum, including *Candida* spp., *C. neoformans*, *Trichosporon* spp., *Aspergillus* spp., *Fusarium* spp. and other hyaline moulds, dematiaceous and dimorphic fungi. The Zygomycetes do not appear to be susceptible (S) to this agent. The oral bioavailability is more than 90%. It is approved for the primary treatment of invasive aspergillosis and infections due to *P. boydii* and *Fusarium* spp. in patients intolerant of, or with infections refractory to, other antifungal agents.

There are other antifungal agents that have been licensed, or are under development for use as therapy of the fungal infections of humans (*e.g.* caspofungin, micafungin, anidulafungin, posaconazole, ravuconazole, *etc.*).

The increasing number and diversity of invasive infections, the expanding utilization of new and established antifungal agents, and the recognition of antifungal

resistance as an important clinical problem have contributed to the need for the reproducible, clinically relevant antifungal susceptibility testing for yeasts and filamentous fungi. The National Committee for Clinical Laboratory Standards (NCCLS) Subcommittee on Antifungal Susceptibility Testing has developed and published approved methods for the broth dilution testing of yeasts (M27-A2), and for the disk diffusion testing of yeasts against fluconazole (M44-A). On the basis of the achievements in standardizing the *in vitro* susceptibility testing of yeasts, the NCCLS antifungal subcommittee has proceeded to develop a standardized method for the broth dilution testing of moulds (NCCLS M38-A).

Different commercially available tests have been developed. These alternative methods most often employ a microdilution format, and are read spectrophotometrically or colorimetrically. Novel breakpoint methods, agar-based methods and flow cytometry have been applied. The Etest (AB Biodisk, Solna, Sweden) is a stable agar gradient minimal inhibitory concentration (MIC) method, which has been shown to be useful in the susceptibility testing of fungi.

II. AIMS OF THE STUDY

1. The identification of yeasts isolated from human samples at a species level is important, but the attainment of this is restricted by the absence of the knowledge and/or the financial possibilities. The aim of this study was to develop schemes for pathogenic yeasts.
2. Participating in the global ARTEMIS Antifungal Surveillance Study (Pfizer) since 1999, we have the possibility to compare our data with the international ones, or to recognize different trends in the antifungal susceptibilities. Our results collected from 2003 to 2005 are evaluated in this presentation.
3. In order to monitor the epidemiology and antifungal susceptibilities of *Candida* species originating from bloodstream infections, the data collected during a 10-year period (1996-2005) are investigated in this study.
4. Despite the fact that blood cultures remain the basic tool for the diagnosis of fungaemia, this method may fail to detect as many as 50% of disseminated cases. The aim of our work was to introduce a molecular genetic method for the detection of human pathogenic fungi in blood samples.
5. Opportunistic filamentous fungal infections have been observed with increasing frequency in recent years, mainly in immunocompromised patients. We evaluated the pathogenic roles of *Fusarium* spp. and *Trichoderma* spp. occurring in human samples. The antifungal susceptibility of filamentous fungal isolates originating from both human samples and the environment is additionally investigated in this study.

III. MATERIALS AND METHODS

1. Specimens

During the 7-year period (from January 1999 to December 2005), all relevant clinical samples submitted for bacterial culturing were screened for fungi. The specimens originated from immunocompetent and immunosuppressed patients cured or hospitalized in different clinical departments of the University Hospital of Szeged and the outpatient care surgeries. Samples from local infections (wounds, smears from mucosal surfaces, *etc.*) were included, and also well as specimens from systemic infections (cerebrospinal fluid, blood, *etc.*).

2. Isolation and identification of fungal strains

All samples (except blood cultures) were inoculated onto Sabouraud chloramphenicol agar (SAB; Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France), and dermatological samples were also inoculated onto Leeming and Notman agar (LNA) plates. The SAB plates were incubated at 37 °C for 1 day, followed by a 6-day incubation at room temperature for the culturing of slow-growing fungi. The LNA plates were incubated at 30 °C for 7 days. Extension of the incubation time to 30 days was considered, depending on the anamnesis, the clinical symptoms and the status of the patient.

The automated blood culture systems (Vital, bioMérieux, Marcy-l'Etoile, France; BACTEC 9120, Becton-Dickinson Diagnostic Systems, Sparks, USA) were used to diagnose the bloodstream fungal infections. All yeast-positive blood samples were subcultured on CHROMagar Candida (CHR; Becton-Dickinson) for rapid identification of the strains, in parallel with SAB plates.

If yeast growth was detected on primarily inoculated agar plates during the incubation period, the fungal strain was identified by the conventional methods (macroscopic /morphology, pigmentation of the colonies, odour of the culture, growing at different circumstances/, microscopic examinations /germ tube test, growth on rice agar, Indian ink staining/, biochemical reactions /urea, esculine hydrolysis, catalase test, tolerance of ethanol/) and/or commercially available media or tests (CHR, AUXACOLOR /BIO-RAD, Marnes-La-Coquette, France/).

Colonies of moulds grown on SAB were examined macroscopically. The texture, the surface and the reverse colour of the colony were noted in addition to any pigment that diffused into the medium and the growth at different temperatures. Various preparation methodologies were used for the microscopic examinations: pressing a portion of the colony with a cover slip in a drop of methylene blue stain, the adhesive tape and/or the slide culture technique. Hydrolysis of urea and *in vitro* hair perforation were carried out according to literature descriptions.

3. Computer-assisted antifungal susceptibility testing of *Candida* spp. isolates

The distributions of yeasts in different clinical samples, susceptibility patterns and trends of activity of fluconazole and voriconazole against isolates have been investigated in the ARTEMIS Antifungal Surveillance Study (Pfizer). Participating in this study, 7089 *Candida* spp. isolates were tested in our laboratory from 2003 to 2005. All strains, considered to be pathogenic from all body sites and isolated from patients in different wards, were included.

Susceptibilities for fluconazole and voriconazole were tested by the NCCLS M44-A method on Mueller-Hinton agar medium supplemented with 2% glucose and 0.5 μg methylene blue per ml, with 25-μg fluconazole and 1-μg voriconazole disks (manufactured by Becton Dickinson, Sparks, Md.). QC strains were: *C. albicans* 90028 from the American Type Culture Collection (ATCC) and *C. parapsilosis* ATCC 22019. Zone diameters were read, interpreted and recorded by using the electronic image-analysis BIOMIC Plate Reader System. The MIC values were calculated automatically with the BIOMIC System software. MIC interpretative criteria for fluconazole were according to the NCCLS M27-A document. For voriconazole, a single preliminary interpretative category of susceptibility exists: MIC ≤ 1 μg/ml.

4. Antifungal susceptibility testing other than BIOMIC

The susceptibilities of 289 *Candida* isolates originating from blood cultures and 50 filamentous fungal strains were determined by the Etest method for fluconazole, itraconazole, ketoconazole and amphotericin B. This was performed in accordance with the manufacturer's instructions, with the use of RPMI 1640 agar supplemented with 2% glucose and Casitone agar plates. Interpretative susceptibility criteria for these antifungal agents were used as published by the NCCLS and in the literature.

The susceptibilities of 10 *Trichoderma* strains were evaluated for fluconazole and amphotericin B by the agar dilution method for amphotericin B and fluconazole on RPMI 1640 agar plates supplemented with 2% glucose. The MIC was read as the lowest drug concentration at which no growth of the microorganisms was detected.

5. Real-time polymerase chain reaction (PCR) method for the detection of fungi in blood samples

From 22 patients with possible fungal bloodstream infection, 54 blood samples treated with the anticoagulant ethylenediaminetetraacetic acid (EDTA) were examined by this method. The preparation of fungal DNA was performed as reported by *Miller et al.* The primers used for universal fungal amplification were internal transcribed spacer (ITS)1, ITS3, ITS4 and ITS86. The size of amplified PCR products varied between 300 and 500 base pairs (bp). The PCR was performed in disposable capillaries with a LightCycler 1.5 (Roche Diagnostics Corporation, Indianapolis, Indiana). PCR amplification was confirmed by the visualization of PCR products by agarose gel electrophoresis.

IV. RESULTS

1. The schemes for identification of human pathogenic yeasts

The exact identification of pathogenic yeasts is important not only from an epidemiologic aspect, but also in the choice of appropriate antifungal agent. Following schemes and tables were developed for the exact identification of yeasts isolated from human samples. Figure 1, table 1 and 2 demonstrate the identification process of yeast isolates with buttery, whitish to cream-coloured, smooth, soft colonies on SAB. Figure 2 and table 3 help in the identification of yeast isolates with wrinkled, cerebriform, dry, farinose, deeply fissured or flat colonies on SAB. *C. krusei*, *C. lambica* and *C. rugosa* can be differentiated by their glucose fermentation, pellicle production in broth and growth at 42 °C. Figure 3 represents the identification scheme of *Malassezia* spp. isolates growing only on LNA, and yeasts producing red-, orange- or salmon-pigmented colonies on SAB can be identified according to the flow chart of Figure 4.

The number of strains identified at a species-level in our mycology laboratory and the number of species identified by the use of these identification schemes grew in 2005 compared with the data for 2002. When we used the new identification schemes for 3720 yeast isolates in 2005, no result was given out without full identification, while in 2002, before this identification process was introduced, 42 strains were reported only as *Candida* species or yeast. The fully identified isolates belonged among 14 species in 2002 and 27 species in 2005.

Figure 1. Scheme for identification of yeast isolates with buttery, whitish to cream-coloured, smooth, soft colonies on SAB

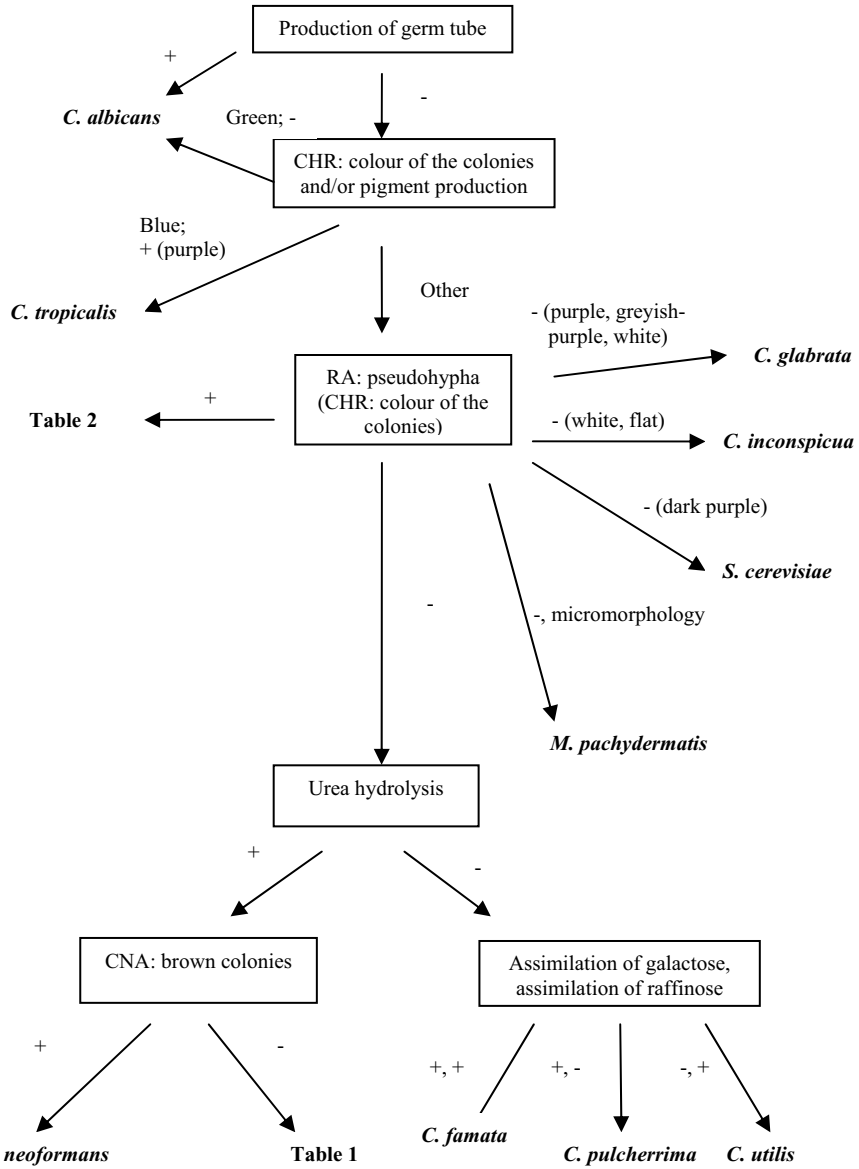


Table 1. Summary of biochemical reactions used to differentiate *Cryptococcus* spp.

Species	Nitrate	Cellobiose	N-Acetyl-D-glucosamine	Melibiose	Ethanol
C. albidus	+	+	-	Various	+
C. ater	-	+	+	-	-
C. curvatus	-	+	+	-	+
C. humicola	-	+	+	+	+
C. laurentii	-	+	-	+	Various
C. uniguttulatus	-	-	+	-	-

Table 2. Carbohydrate assimilation and growth of different *Candida* species producing pseudohyphae on 0.1% cycloheximide-containing medium

Species	0.1% cycloheximide	Cellobiose	Galactose	Lactose	Maltose	Raffinose
C. guilliermondii	+	+	+	-	+	+
C. haemulonii	+	-	Slow	-	+	+
C. intermedia	Various	+	+	+	+	+
C. kefyr	+	Various	Slow	Various	-	+
C. lusitaniae	-	+	+	-	+	-
C. norvegensis	-	-	-	-	-	-
C. parapsilosis	Various	-	+	-	+	-
C. pelliculosa	-	+	+	-	+	+
C. rugosa	-	-	+	-	-	-
C. viswanathii	+	+	+	-	+	-

Figure 2. Scheme for identification of yeast isolates with wrinkled, cerebriform, dry, farinose, deeply fissured or flat colonies on SAB

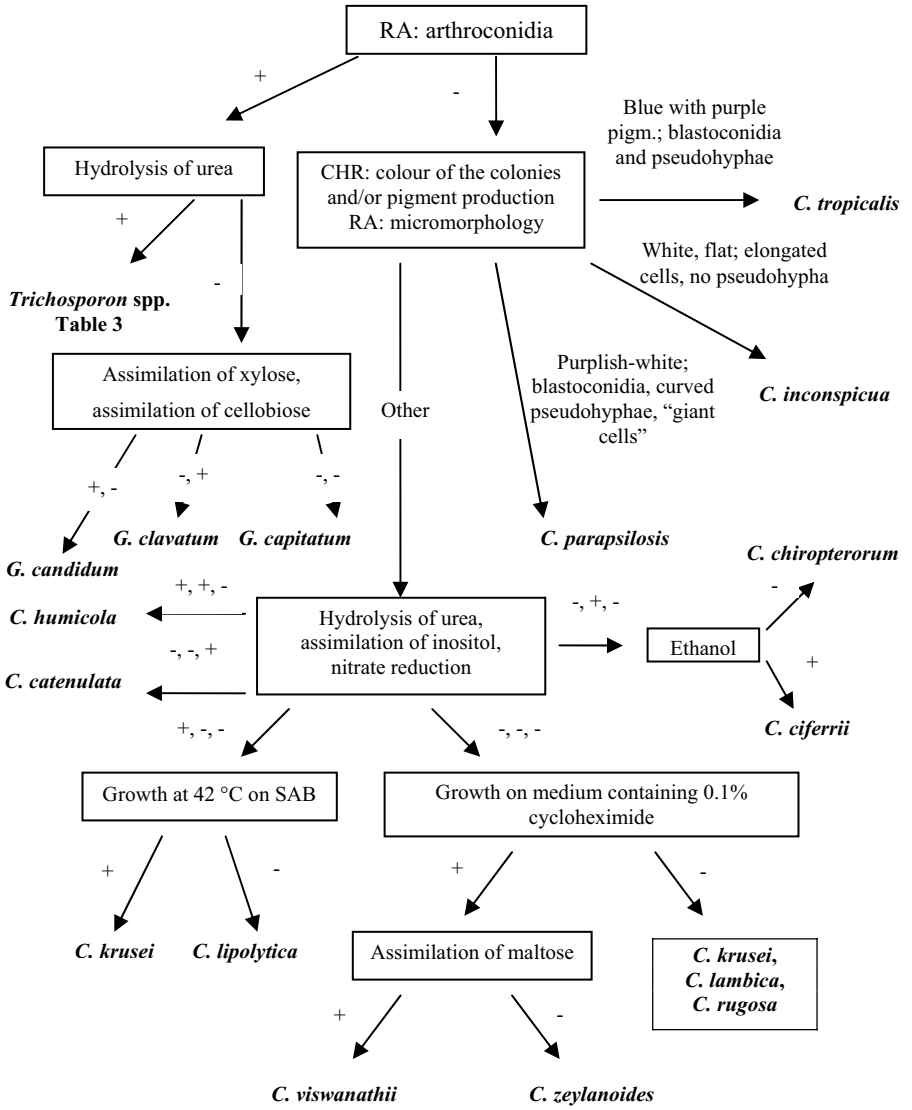


Table 3. Assimilation and morphological features of *Trichosporon* species

Species	Arabinose	Inositol	Growth at 37 °C	Appressoria
T. asahii	+	-	+	-
T. cutaneum	+	+	-	-
T. inkin	-	+	+	+
T. mucoides	+	+	+	-
T. ovoides	Various	-	Various	+

Figure 3. Scheme for identification of *Malassezia* spp. isolates growing only on LNA

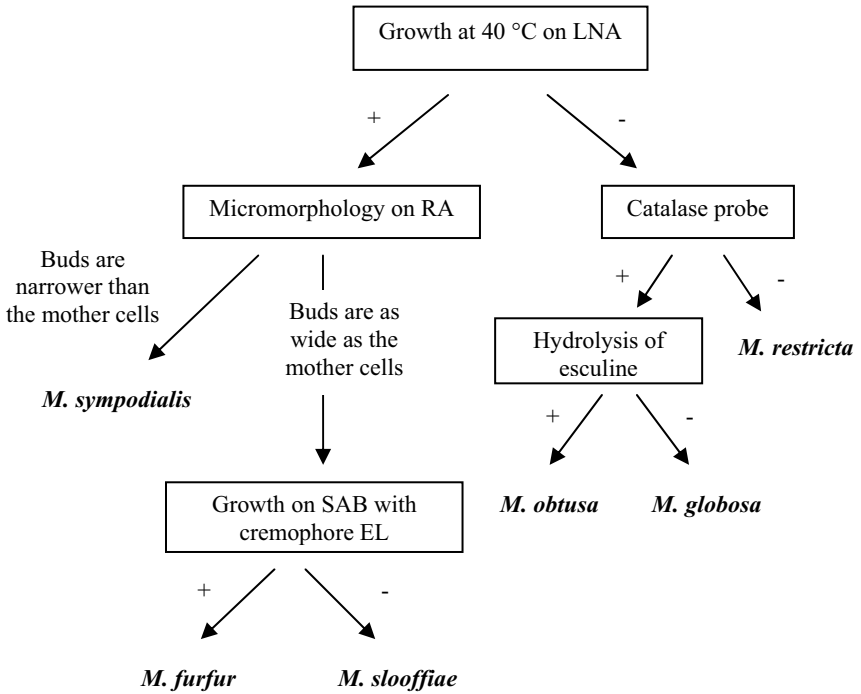
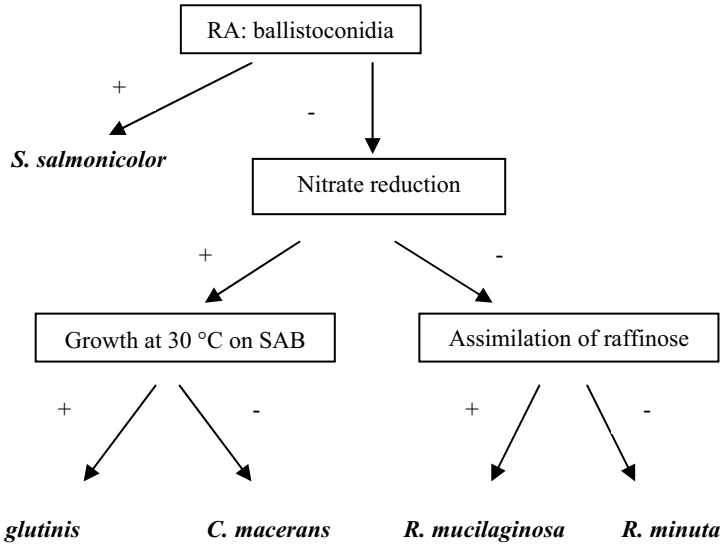


Figure 4. Flow chart for the identification of yeasts producing red-, orange- or salmon-pigmented colonies on SAB



2. Computer-assisted antifungal susceptibility testing of *Candida* spp. isolates

During the 3-year period (2003-2005), 7089 *Candida* spp. strains were tested within the frame of the study. The total numbers of isolates were similar in 2004 (n = 2077) and 2005 (n = 2147), but the number in 2003 was higher (n = 2865). The rates of tested *C. albicans* and *C. glabrata* (the most frequently isolated species) were similar in every year: close to 80% and 9%, respectively. The number of *C. krusei* was higher (n = 99), than that of *C. tropicalis* (n = 61) in 2003, but the difference decreased in 2004, and increased in 2005 in favour of *C. tropicalis*. Thus, *C. tropicalis* has become the third most frequently tested species. The rates of *C. inconspicua* and *C. kefyr* remained between 1 and 2% during the 3-year period. More *C. parapsilosis* isolates were tested than *C. lusitaniae*. The testing rate of *C. lusitaniae* remained below 1%, while in the case of *C. parapsilosis* it increased slightly above 2%.

The majority (more than 99%) of the *C. albicans* strains were S to fluconazole by the disk diffusion method. The cumulative rates of SDD and R *C. albicans* isolates were smaller than 0.5% in every year. The proportion of fluconazole-S *C. glabrata* strains was higher (83.8%) in 2004, than in the other two years. 45-61% of the *C. inconspicua* strains proved R to this antifungal agent, while the corresponding interval for *C. krusei* was 83-91%. Fluconazole-S *C. krusei* isolates were not found. The rate of S *C. tropicalis* has been decreasing since 2003 (95.1%, 88.9% and 84.5%). Among the species isolated with relatively low frequency (other *Candida* spp.), there were more R than S and SDD together in 2003 and 2004. The proportions of other fluconazole-S and –R *Candida* spp. strains were similar (58.3% and 41.7%) in 2005.

The efficacy of voriconazole against *Candida* spp. is revealed by its MIC50 and MIC90 values. These data are available only from the annual summary report for the whole

of Hungary, not only for our laboratory. The voriconazole data obtained in 2004 and 2005 are not yet available. The MIC values obtained for voriconazole in Hungary in 2003 are compared with MICs for fluconazole in Table 4. The voriconazole MIC 50 and MIC 90 of *C. albicans*, *C. kefyr*, *C. lusitaniae* and *C. parapsilosis* were lower than in the cases of fluconazole-R strains (*C. glabrata*, *C. inconspicua*, *C. krusei*). The voriconazole MIC values obtained by the disk diffusion method for *C. tropicalis* were rather similar to the MICs of the fluconazole-R species, though *C. tropicalis* is a fluconazole-S species. Among fluconazole-R species, *C. glabrata* has the highest voriconazole MIC90, exceeding the preliminary interpretative category of susceptibility, MIC ≤ 1 μg/ml. However, the fluconazole MIC values for *C. glabrata* were lower than in the cases of *C. krusei* and *C. inconspicua* in 2003 (Table 4).

Table 4. Comparison of fluconazole and voriconazole MICs (MIC 50 and MIC 90; μg/ml) of the most frequently isolated *Candida* spp. in 2003

Organisms	Fluconazole		Voriconazole		N
	MIC 50	MIC 90	MIC 50	MIC 90	
C. albicans	0.5	4	0.032	0.125	3094
C. glabrata	16	64	0.5	2	459
C. inconspicua	128	> 256	0.25	1	59
C. kefyr	0.125	2	0.016	0.064	47
C. krusei	> 256	> 256	0.5	1	171
C. lusitaniae	1	8	0.016	0.25	25
C. parapsilosis	2	64	0.032	0.5	62
C. tropicalis	4	32	0.25	1	171

3. Investigation of the prevalence and antifungal susceptibilities of *Candida* isolates originating from bloodstream infections

During the evaluated period (from 1996 to 2005), 316 *Candida* spp. strains were isolated: 190 *C. albicans* (60.1%), 55 *C. glabrata* (17.4%), 25 *C. parapsilosis* (7.9%), 17 *C. krusei* (5.4%), 15 *C. tropicalis* (4.7%), 6 *C. inconspicua* (1.9%), 4 *Candida* spp. (1.3%), 2 *C. kefyr* (0.6%) and 2 *C. sake* (0.6%). In some cases, the same species was isolated from more than one blood culture bottle from the same patient. There were 4 patients, whose candidaemia was caused by two different species: 1 *C. albicans* and *C. kefyr*, 1 *C. parapsilosis* and *C. glabrata*, 1 *C. glabrata* and *C. albicans*, and 1 *C. albicans* and *C.*

parapsilosis. Most of the *Candida* spp. isolates were from patients hospitalized in intensive care units (ICUs): 97 (30.7%) were obtained from adult ICUs, and 82 (25.9%) from paediatric ICUs. Fewer isolates originated from blood cultures from patients hospitalized in adult surgical wards (67; 21.2%) and other departments (including haematology and cardiology; 47; 14.9%). Altogether, 133 patients had a bloodstream infection caused by different species of *Candida* during this 10-year period. The numbers of episodes were similar in paediatric and adult ICUs (38 and 40 episodes), and in adult internal and surgical departments (27 *vs* 23).

The isolates have been tested for their susceptibilities to fluconazole, itraconazole, ketoconazole and amphotericin B since 1997. Among the *C. albicans* isolates, 92.6% and 82.8% were S to fluconazole and itraconazole, respectively. The rates of isolates R to these azole antifungal agents were similar (4%), but more strains were SDD to itraconazole (13.2%) than to fluconazole (3.4%). More *C. glabrata* isolates were S to fluconazole (n = 43) than to itraconazole (n = 15). All of the tested *C. krusei* strains were R to both antifungal agents. Another species known to be fluconazole-R, *C. inconspicua*, was S to itraconazole, while the MICs obtained for fluconazole were in the SDD range. *Candida* spp. bloodstream isolates tested for ketoconazole (65 *C. albicans*, 51 *C. glabrata*, 12 *C. parapsilosis*, 10 *C. tropicalis*, 6 *C. inconspicua*, 2 *C. sake*, 2 *Candida* spp. and 1 *C. krusei*) were S (MIC < 8 μg/ml), with the exception of 1 *C. glabrata* strain, which was R to this antifungal agent, with MIC = 32 μg/ml. Two other *C. glabrata* strains originated from a patient whose third isolate was fully R to the azole antifungal agents tested (mentioned previously): the fluconazole MIC changed from 32 μg/ml to 256 μg/ml, the itraconazole MIC remained at 32 μg/ml, and the ketoconazole MIC increased from 1 μg/ml to 32 μg/ml. All the *Candida* isolates tested for amphotericin B (77 *C. albicans*, 47 *C. glabrata*, 18 *C. parapsilosis*, 8 *C. tropicalis*, 6 *C. inconspicua*, 4 *C. krusei*, 2 *Candida* spp. and 2 *C. kefyr*) were S, with MIC < 1 μg/ml.

4. Real-time PCR method for the detection of fungi in blood samples

Fifty-four blood samples treated with anticoagulant (EDTA) originating from 22 patients (aged from 2.8 to 81 years; mean 52.8 years) with a possible fungal bloodstream infection were examined. The ratio of females and males was 13/9. The patients involved in this study were immunocompromised, and/or their symptoms suggested the suspicion of bloodstream infection or sepsis caused by fungus (*e.g.* the same fungal species were cultured from different clinical specimens). The first group of patients (13 patients) gave no positive samples either by PCR or in their blood cultures. Among them, 4 patients were treated with fluconazole: 1 patient pre-emptively and 3 patients empirically. The mortality rate in this group was 38%. There was a 20-year old girl (patient 14) with polytrauma, a respiratory insufficiency and septicaemia, whose blood cultures were positive, and the blood sample collected for PCR on the same day was negative for fungi. Her polymicrobial (bacterial and fungal) septicaemia was treated successfully, and she survived. The third group contains 3 patients (15-17) who also had positive blood cultures at times near to when their PCR tests were positive for fungi. Blood samples for PCR were collected in view of blood culture positivity in the cases of 15 and 16 patients. The last group contains 5 patients (18-22) with positive PCR results and without blood culture positivity for fungi. The mortality in this group was 1 out of the 5 patients. All these patients were treated with antifungal agents.

5. Clinical importance of environmental filamentous fungi in human infections

Fusarium spp. are commonly found in soil, in marine or river environments, and on plants throughout the world. Fusariosis occurs most frequently as a localized infection of the cornea: corneal trauma is the most common predisposing factor for keratomycosis. In our own experience, a case of keratomycosis caused by a mixed infection involving *F. verticillioides* was diagnosed in a 47-year-old Hungarian man. In addition to a left eye trauma caused by a stone during motorcycling, another potential predisposing factor was his untreated diabetes. After the injury, the corneal epithelial lesion developed into a severe visual impairment, despite local antibiotic (ciprofloxacine) and steroid (prednisolone and dexamethasone) treatment. After 4 weeks, a dense, mesh-like structure in the anterior chamber, attached to the posterior surface of the cornea, was revealed by ultrasound biomicroscopy. Local natamycin (pimaricin) therapy was started, but the mesh-like structure progressed, filling almost the entire anterior chamber. Penetrating keratoplasty was performed as the only possible treatment. Intraoperative samples taken from the cornea and the anterioir chamber yielded bacteria (*Staphylococcus epidermidis*, an α-haemolytic Streptococcus and *Propionibacterium acnes*) and *F. verticillioides*. The identification of *F. verticillioides* was based on its macro- and micromorphological properties. Local steroid (prednisolone) and antibiotic (neomycin and tobramycin) treatment, combined with oral itraconazole therapy, was started postoperatively. The diabetic status improved following the initiation of insulin therapy. Three months later, the host and graft cornea were transparent without inflammatory signs. The best-corrected vision was 0.9.

The members of the *Trichoderma* genus can be found mainly in the soil, but they can also occur on wet surfaces (walls of the rooms, old papers deposited in wet rooms, *etc.*). However this genus is also on the growing list of potential fungal pathogens in immunocompromised hosts. The predisposing factors are similar to those of *Fusarium* spp. Seven *Trichoderma* spp. strains (*T. citrinoviride* 9573 from the University of Alberta Microfungus Collection and Herbarium /UAMH/, *T. koningii* 382 from the Centro Nacional de Microbiologia, Immunologia y Virologia Sanitaria /CM/, *T. longibrachiatum* ATCC 201044, ATCC 208859, UAMH 7955, UAMH 7956 and UAMH 9515) originating from human samples were investigated not only from ecophysiological and enzymological aspects, but also for their resistance to antifungal drugs. These strains originated from different type culture collections. All of these strains were able to grow in a wide range of temperature (from 10 °C to 40 °C), with growth optimum at 30 °C. A pH ranging from 2.0 to 9.0 supported the growth of most of the strains examined at 25 °C, with the optimum at pH 4.0. At elevated temperature (37 °C), the range was narrower, but all of the clinical strains were able to grow at physiological pH. These factors are basic for their opportunistic pathogenic role. This made possible the evaluation of the results of their susceptibility testing for antifungal agents used in the treatment of the diseases they caused.

6. Evaluation of antifungal susceptibility of filamentous fungi originating from the human samples and the environment

During a 3-year period (2000-2002), 50 filamentous fungi originating from different specimens were evaluated. Thirty-two human isolates were cultured from specimens from the upper or lower respiratory tract. Three isolates were from the peritoneal fluid, 4 from the gastrointestinal tract, 3 from mucocutaneous samples and 2 from the genital

tract. The filamentous fungal strains tested for their susceptibilities to fluconazole, ketoconazole, itraconazole and amphotericin B were *Acremonium* spp. (1), *Aspergillus candidus* (1), *A. fumigatus* (4), *A. niger* (5), *A. ochraceus* (2), *A. versicolor* (6), *Cladosporium* spp. (2), *Penicillium chrysogenum* (6), *P. humicola* (2), *P. humuli* (4), *Penicillium* spp. (3), *Scopulariopsis* spp. (1), *Trichoderma citrinoviride* (1), *T. koningii* (1) and *T. longibrachiatum* (5) originating from human specimens, and *Epicoccum nigrum* (1), *Scopulariopsis* spp. (1), *T. citrinoviride* (1), *T. koningii* (1), *T. longibrachiatum* (1) and *T. pseudokoningii* (1) from environmental samples. Forty-six of the tested moulds (92%) were R to fluconazole, with MIC > 256 μg/ml. The range of MICs for ketoconazole was wide: there were R strains with MICs > 32 μg/ml among the *A. niger*, *A. ochraceus* and *Cladosporium* spp., while the lowest MIC values were obtained for the *Trichoderma* strains (0.008−0.5 μg/ml). Two strains (1 *A. ochraceus* and 1 *P. chrysogenum*) were R to amphotericin B, with MIC values > 32 μg/ml.

For 10 selected isolates, the MICs obtained by the Etest were measured by using two different spore suspension turbidities. For fluconazole, no differences were observed with the two inocula: all the strains were fully R, with MIC > 256 μg/ml. For itraconazole, ketoconazole and amphotericin B, a 1- or 2-step 2-fold dilution difference in MIC was seen for most strains, with the exception of *A. niger* 2 and *A. ochraceus*: significant differences in MICs were detected for ketoconazole and itraconazole in the cases of these two strains.

The MICs of fluconazole and amphotericin B obtained for 10 *Trichoderma* strains by the Etest and the agar dilution method were compared. The MICs of fluconazole obtained with the two methods were in agreement for 8 strains, but higher values were obtained by the Etest for the strain *T. koningii* T 39. Higher MICs were obtained for amphotericin B with the agar dilution method than with the Etest in the cases of 7 strains; however, most of these differences were within ± 2 two-step dilutions.

V. DISCUSSION

1. Identification schemes for human pathogenic yeasts

The most frequent genus occurring in clinical samples is *Candida*, and the most frequent species is *C. albicans*. It can be identified by several (micro-, macromorphological, biochemical and molecular) methods, but the cheapest and the most rapid is the examination of germ tube production. *C. tropicalis* was reported to form germ tubes, but this ability was rapidly lost during repeated subculturing. The rate of germ tube-producing *vs* budding cells is much smaller for *C. tropicalis* than for *C. albicans*. If the investigator has practice in the evaluation of this method, these species should not be confused. The phenotypic characteristics of *C. dubliniensis* are very similar to those of *C. albicans*: it produces germ tubes, chlamydospores with pseudohyphae, green colonies on CHR, *etc.* There have been several reports on the evaluation of phenotypic markers, but the reliable differentiation of these two species seems to be based on molecular genetics.

CHR is a good medium for the differentiation of yeast species from mixed cultures, for the identification of *C. albicans* and *C. tropicalis*, and for the presumptive identification of *C. glabrata*, *C. krusei*, *C. kefyr*, *C. parapsilosis*, *C. inconspicua* and *S. cerevisiae*. Additional information (micromorphology and growing conditions) is needed for the exact identification of the last 6 species. The purple pigmentation in the CHR medium is visible sooner than the typical blue colour of colonies of *C. tropicalis*.

The micromorphology on RA is one of the most reliable methods of identifying human pathogenic yeasts (not only the members of *Candida* genus), but its evaluation

demands considerable practice. However, in some cases the isolates do not exhibit the typical morphology. The cause of this may be the antimicrobial and immunosuppresive therapy of the patient. In this case, the result should be verified by other methods.

The colour changes of the indicators of biochemical tests depend on the environmental circumstances (*e.g.* acidity in the air of the laboratory). Accordingly, these influencing factors should be minimized by application high level of prudence.

Yeasts other than *Candida* spp. are included in the schemes because they may also be obtained as pathogens with different frequency from human samples, mainly in the cases of immunosuppressed patients.

Although the number of yeast isolates was higher in 2005 than in 2002, more isolates were identified at a species level in 2005. The rate of isolates that were not identified at a species level decreased from 1.4% to 0 during the interval 2002-2005, while the schemes were developed. The number of identified species increased from 10 to 27.

2. Fluconazole and voriconazole susceptibilities of *Candida* spp. isolates

The rate of tested *C. albicans* strains was higher, and that of *C. glabrata* was lower during the 3-year period (2003-2005) in our laboratory than the corresponding rate in Hungary from 2001 to 2003. Although a decreasing trend in the rate of *C. albicans* isolation was reported on the basis of global data between 1997 and 2001, our percentages obtained from 2003 to 2005 were more than 15% higher. The rate of *C. glabrata* was 11% of the total number of *Candida* spp. strains tested in Hungary in 2004. Thus, the lower rate obtained in our laboratory since 2003 is not a result of a decreasing tendency in Hungary. This is in contrast with *C. krusei*, because its rates were 4% of all *Candida* spp. isolates tested in 2003 and 3% in 2004 in Hungary, while in our laboratory these rates were 4% in 2003 and 2% in 2004 and 2005. This may be caused by a decreasing trend in the number of *C. krusei* isolates. During the 3-year period, the rate of isolation of *C. parapsilosis* and *C. tropicalis* remained below the global values obtained in 2001 (6 and 7% respectively), and an increasing trend was reported.

The fluconazole susceptibility results obtained for *C. albicans* isolates in our laboratory were very similar to the Hungarian ones in 2003. A possible cause may be that almost 75% of the total number of Hungarian *C. albicans* isolates tested in the ARTEMIS Antifungal Surveillance Study in this year originated from our laboratory. The situation is similar in the cases of the *C. inconspicua*, *C. kefyr* and *C. lusitaniae* species. The higher rate of fluconazole-S *C. glabrata* isolates obtained in 2004 may be caused by the smaller number of tested strains. Among the species known to be fluconazole-R, SDD and/or S, isolates were detected in smaller (*C. krusei*) or in higher (*C. inconspicua*) percentages by the disk diffusion method. According to the literature data, voriconazole exhibited markedly enhanced *in vitro* activity against most of the yeast isolates originating from different human specimens. In our study, the voriconazole MIC 90 was near the limit of the preliminary interpretative category of resistance (> 1 μg/ml) in the cases of the fluconazole-R *Candida* species (mainly *C. glabrata*) and the fluconazole-S *C. tropicalis* in 2003 (Table 4). A final revision of the susceptibility and resistance breakpoints for voriconazole would be desirable in view of the clinical data.

3. Prevalence and antifungal susceptibilities of *Candida* isolates originating from bloodstream infections

This study evaluated the occurrence of bloodstream infections caused by *Candida* spp. among patients hospitalized in different clinical wards of the University Hospital of Szeged from 1996 to 2005. During this 10-year period, a total of 133 episodes of candidaemia were detected in various numbers (between 5 and 31) in each year. The total number of episodes was highest in 2005, because 12 infants hospitalized in paediatric ICUs were affected by an epidemic caused by *C. glabrata*. The number of episodes caused by *C. albicans* was highest every year. However, the numbers of episodes caused by *C. albicans* and *C. glabrata* were equal in 2005. In the same year, more cases were caused by the isolated non-*albicans Candida* species than by the same species in previous years. The incidence of *C. glabrata* isolated from bloodstream infections has been rising: this species has been isolated every year since 2003. The frequency of isolation of *C. parapsilosis* was constant, while the incidence of *C. krusei* decreased during the evaluated period. *C. inconspicua*, *C. kefyr* and *C. sake* occurred sporadically.

Interesting similarities and differences can be seen in the studies from different countries inside and outside Europe. The first three species originating from bloodstream infections were *C. albicans*, *C. tropicalis* and *C. parapsilosis* in Turkey, *C. albicans*, *C. parapsilosis* and *C. tropicalis* in Italy, Spain, Israel and Southern Brazil, *C. albicans*, *C. glabrata* and *C. tropicalis* in Denmark, and *C. albicans*, *C. glabrata* and *C. parapsilosis* in France and Hungary. The rate of bloodstream infections caused by two different *Candida* species at one time was slightly higher in our study (3%) than in Spain (2%).

The total numbers of septic episodes caused by *Candida* spp. were similar in the paediatric and adult ICUs (38 and 40, respectively), but the number of bloodstream infections caused by *C. glabrata* was about 3-fold higher in the paediatric ICUs. The possible cause of this was the epidemic in 2005. Candidaemia caused by a member of the normal human skin flora, *C. parapsilosis*, occurred in every department, but the numbers of episodes caused by this species did not differ markedly. As regards the total numbers of episodes, the adult internal and surgical departments gave similar results.

Out of 287 *Candida* isolates tested for fluconazole susceptibility, 240 (83.6%) were S to this antifungal agent. The fluconazole resistance was 4% among the *C. albicans* strains. This rate is higher than reported from other countries: 1.6% in Israel, 1% in the countries participating in the SENTRY Program (U.S.A., Canada, Latin America and Europe) and 0 in Spain. The rates for *C. albicans* strains found to be SDD or R to fluconazole were similar. The percentages of fluconazole-SDD and –R *C. glabrata* isolates were 20% and 1.8%, respectively. According to the Spanish surveillance, the rate of isolates SDD to fluconazole was slightly smaller, at 16%, and the percentage of resistance was 3%. Azole cross-resistance was detected in one case caused by *C. glabrata*. Nearly half of the *C. tropicalis* isolates tested possessed a decreased susceptibility to fluconazole *in vitro*.

The rate of resistance to itraconazole (14.3%) was twice as high as that to fluconazole (7.3%). This was similar to data reported in 1997 from different parts of America, but the percentages were lower (3.6% for fluconazole and 8.5% for itraconazole) than in our laboratory. More *C. albicans*, *C. glabrata* and *C. parapsilosis* isolates were SDD or R to itraconazole than to fluconazole, but adverse results were obtained for the *C. tropicalis* strains. All of the *C. inconspicua* isolates SDD to fluconazole were S to itraconazole.

4. Real-time PCR method for the detection of fungi in blood samples

Blood samples originating from patients with different predisposing factors were examined by a real-time PCR method. Patients 1 and 2 gave no samples that were positive for fungi. Their inclusion in the study was not clinically reasonable. Patients with one or more different samples positive for fungi occurred in the first group; they gave no positive blood samples. The assumption of fungaemia or fungal sepsis was not confirmed microbiologically. This was proved by the fact that 5 of the 7 untreated patients and 2 of the 4 patients treated with fluconazole survived. The mortality rate was higher among the treated patients. Bacterial sepsis or the underlying disease(s) of the patients may have been responsible for the relatively high mortality rate in the first group, containing 13 patients.

In the case of patient 14, different results were obtained by culturing and the PCR method for her blood samples collected on the same day. A possible cause of this may be the cell count in the samples in connection with the examined quantities of blood. Twenty-ml blood samples are needed in blood culture bottles used for adults. The preparation for the PCR reaction started with a 500-µl specimen. The DNA originating from the fungi was resuspended in 200 µl elution buffer, and 1 µl of this solution was used for the PCR reaction. If the cell count is small, it may happen that there is no target DNA in the equal amount of blood sample. Two possibilities are available to resolve this problem. The first is at the starting point: a higher amount of sample (*e.g.* 1 ml) should be used for the preparation. However, this needs laboratory tools different from those used for PCR reactions (*e.g.* tubes and centrifuges). The other solution may relate to the step of DNA resuspension: the DNA should be resuspended in a smaller quantity (*e.g.* 50 µl) of elution buffer. This may result in concentration of the DNA solution used for the PCR reaction so that it achieves the limit of sensitivity of the PCR reaction (5 colony-forming unit/ml).

In the cases of patients 15 and 16, the culturing results were confirmed by the real-time PCR method, because the blood samples for PCR were collected in view of positive blood cultures. However, the utility of this method is also apprehended in the case of patient 17. Of his 9 blood samples taken during a 13-day period for the PCR detection of fungi, 2 were positive. Unfortunately, the antifungal therapy (amphotericin B) was started only when the blood cultures had also become positive, the clinician thereby missing the opportunity to use the earlier detection of the fungal DNA in the blood. If this patient had received adequate antifungal therapy in time (*e.g.* after the first PCR-positive sample), his possibly transient fungaemia would not have developed into a fungal bloodstream infection.

The advantage of the PCR methodology is demonstrated in the fourth group of patients. Although their blood cultures (or other specimens) were negative for fungi, almost 100% of their blood samples were positive for fungi by the PCR method. Their antifungal treatment could be started in time on the basis of the positive PCR results, and thus the mortality rate in this group was better (1 out of 5 patients) than that mentioned previously.

The real-time PCR introduced in our laboratory affords a possibility for the rapid detection of fungi in blood samples. The benefits of this method (antifungal therapy started in time, and a shorter nursing time) compensate its costs. It can be started on smaller amounts of blood samples, which is useful mainly in the cases of paediatric patients.

5. Roles of *Fusarium* and *Trichoderma* species in human infections

Members of the *Fusarium* genus occur in the soil all over the world. A review of the literature published in connection with this filamentous fungal genus revealed that it is among the agents that most frequently cause keratomycosis in humans. Fusarial keratitis is most common among agricultural workers in geographical regions with hot, humid, tropical or semi-tropical climates. This genus was the predominant aetiological agent of keratomycosis in many surveys, but the incidence may vary with the climatic conditions within a single country. However, fusarial keratitis is rare in European countries with temperate climates: 4 cases have been reported from Paris, France during an 8-year period. The species reported most often was *F. solani*. Corneal trauma is the most common predisposing factor. The injuries are caused by various traumatizing agents (plant material, animal matter, dust, soil, mud, stones, glass, metal objects and fingernails). Other predisposing factors may be the use of topical corticosteroids, previous eye surgery, pre-existing ocular diseases, systemic diseases (*e.g.* diabetes mellitus) and the wearing of contact lenses. The first sign of fungal keratitis may be the presence of a coarse granular infiltration of the corneal epithelium and the anterior stroma. However, this symptom also occurs with bacterial keratitis. A possible complication may be endophthalmitis, which may result in the deterioration of visual acuity, including the loss of light perception.

Trichoderma species are also potential opportunistic pathogenic filamentous fungi. They have different virulence factors, which may play important roles in causing human infections (*e.g.* growth at elevated temperature /37 °C/, tolerance of neutral pH). All of the examined clinical strains were able to grow under the circumstances mentioned above. Other ecophysiological studies of *Trichoderma* genus have revealed that species belonging in the Longibrachiatum section have higher optimum growth temperatures. This may be the reason why most of the strains involved in *Trichoderma* infections belong in the Longibrachiatum section of the genus. The utilization of amino acids as sole sources of both carbon and nitrogen may serve as a potential virulence factor, as may their low-level susceptibilities to antifungal drugs. The level of their protease production is higher at 37 °C than at 25 °C under similar inductive circumstance (shaking).

6. Antifungal susceptibility testing of filamentous fungi originating from human samples and the environment

The susceptibility testing of filamentous fungal isolates is important when the risk of invasive infection exists. In this study, most strains were R to fluconazole by the Etest, while ketoconazole, itraconazole and amphotericin B were more effective. For most strains, the MICs were not influenced significantly by the turbidity of the spore suspension. For 8 of 10 strains, there were no or only small differences between the MICs obtained on the use of 3 McFarland and 0.5 McFarland suspensions. If the higher inoculum was used for some slow-growing isolates, the MICs could be read 1 day earlier. Higher differences were detected for 2 *Aspergillus* strains: *A. niger* and *A. ochraceus*. The MICs of these strains for amphotericin B and fluconazole did not differ markedly, and were similar to the itraconazole MIC of *A. ochraceus*. These moulds grew rapidly and covered the surface of the agar plates completely (with the exception of the inhibition zone) when a higher turbidity of suspension was used. The cultures were thicker, which influenced the MICs. More exact values could be obtained if the turbidity of the spore suspension was measured spectrophotometrically in the case of black moulds.

The differences in MIC values obtained by comparison of the Etest and the agar dilution methods were similar for the evaluated *Trichoderma* strains. The MICs for fluconazole did not differ markedly with these methods, but they did differ by 1 or 2 steps of 2-fold dilutions from the data in the literature obtained by broth dilution methods. The MICs

for amphotericin B were higher by 1 or 2 steps of 2-fold dilutions with the agar dilution method.

These data indicated differences between the susceptibility testing methods for moulds. The agar dilution method was not the most appropriate for the susceptibility testing of moulds in routine laboratory practice, because the exact results depended on numerous features (*e.g.* the stability of the solution of antimycotics, the mixing of the stock solutions into the agar medium, the growth rate of the moulds, *etc.*). The Etest is easier to perform: it is less labour-intensive, it is much simpler to set up than the broth microdilution or agar dilution methods and it provides the flexibility to test antifungal agents against different moulds.

VI. CONCLUSIONS

1. We have developed identification schemes for yeasts isolated more or less frequently from human specimens. These schemes make possible the exact identification of these yeasts at a species level by using macro- and micromorphological examinations, physiological investigations and biochemical reactions.
2. Participating in the international ARTEMIS Antifungal Surveillance Study, we collected fluconazole and voriconazole susceptibility data, obtained by the disk diffusion of yeast strains isolated from human specimens. Trends or changes in the antifungal susceptibilities can be followed by this method, as many *C. glabrata* and *C. inconspicua* isolates are S to fluconazole. It can be observed in the case of *C. tropicalis* isolates that the ZD around the 1-µg voriconazole disk is smaller than that around the 25-µg fluconazole disk, but the reverse holds true for any other *Candida* species known to be fluconazole S.
3. For the first time in Hungary, we have investigated and published the aetiology and antifungal susceptibility data on yeast isolates originating from blood cultures. Our aetiological and antifungal susceptibility data collected during a 10-year period are comparable with others originating from other countries.
4. We introduced and optimized a DNA preparation method and a real-time PCR method, suitable for the detection of fungi in blood samples. Results can be obtained with this process within 3 h, whereas the culturing needs at least 24 h.
5. For the first time in Hungary, we published a case of fungal keratitis caused by *F. verticillioides*. We summarized the clinical importance of *Fusarium* spp. in keratitis. The connection between the ecological and physiological properties of *Trichoderma* spp. and the epidemiology of the infections they cause was also revealed. We found a relationship between the *Trichoderma* species (*T. longibrachiatum*) most frequently isolated from human samples and the ecological properties of the Longibrachiatum section of this genus.
6. Antifungal susceptibilities as potential virulence factors of filamentous fungi with different origins were compared in this study. The results obtained under different testing circumstances and different susceptibility testing methods were evaluated. For most strains, the MICs were not influenced significantly by the turbidity of the spore suspension. Our results suggest that the Etest is more appropriate than the broth and agar dilution methods for the susceptibility testing of moulds in routine laboratory practice.

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