Department of Clinical Microbiology, Faculty of Medicine
University of Szeged, Szeged, Hungary

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

Ilona Dóczi

Supervisor: Prof. Dr. Elisabeth Nagy

2006

Szeged

CONTENTS

COMMUNICATIONS CONNECTED WITH THE THESIS

I. **Ilona Dóczi,** Gyula Mestyán, Erzsébet Puskás, Radka Nikolova, István Barcs, Elisabeth Nagy:
Use of the BIOMIC Video System to evaluate the susceptibility of clinical yeast isolates to fluconazole and voriconazole by a disk diffusion method
Acta Microbiol Immunol Hung (Accepted for publication)

II. **Ilona Dóczi**, Erika Dósa, Edit Hajdú, Elisabeth Nagy:
Aetiology and antifungal susceptibility of yeast bloodstream infections in a Hungarian university hospital between 1996 and 2000
J Med Microbiol, **51:** 677-681 (2002) **I.F.: 1.987**

III. Erika Dósa, Ilona Dóczi, László Mojzes, Etelka Molnár G., János Varga, Erzsébet Nagy:
Identification and incidence of fungal strains in chronic rhinosinusitis patients
Acta Microbiol Immunol Hung, **49:** 337-346 (2002)

IV. László Kredics, Zsuzsanna Antal, **Ilona Dóczi**, László Manczinger, Ferenc Kevei, Elisabeth Nagy:
Clinical importance of the genus *Trichoderma*. A review.
Acta Microbiol Immunol Hung, **50:** 105-117 (2003)

V. **Ilona Dóczi**, Tamás Gyetvai, László Kredics, Elisabeth Nagy:
Involvement of *Fusarium* spp. in fungal keratitis
Clin Microbiol Infect, **10:** 773-776 (2004) **I.F.: 2.238**

VI. Zsuzsanna Antal, László Kredics, Jaako Pakarinen, **Ilona Dóczi**, Maria Andersson, Mirja Salkinoja-Salonen, László Manczinger, András Szekeres, Lóránt Hatvani, Csaba Vágvölgyi, Elisabeth Nagy:
Comparative study of potential virulence factors in human pathogenic and saprophytic *Trichoderma longibrachiatum* strains
Acta Microbiol Immunol Hung, **52:** 341-350 (2005)

VII. **Ilona Dóczi**, Erika Dósa, János Varga, Zsuzsanna Antal, László Kredics, Elisabeth Nagy:
Etest for assessing the susceptibility of filamentous fungi
Acta Microbiol Immunol Hung, **51:** 271-281 (2004)

ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
ATCC	American Type Culture Collection
bp	base pairs
CF	cystic fibrosis
CHR	CHROMagar Candida
CM	Centro Nacional de Microbiologia, Immunologia y Virologia Sanitaria
CNA	*Cryptococcus neoformans* agar
DNA	desoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ICU	intensive care unit
ITS (1, 3, 4, 86)	internal transcribed spacer (1, 3, 4, 86) regions
LNA	Leeming and Notman agar
MIC	minimal inhibitory concentration
NCCLS (CLSI)	National Committee for Clinical Laboratory Standards (Clinical and Laboratory Standards Institute)
PCR	polymerase chain reaction
QC	quality control
R	resistant
RA	rice agar
rpm	rotations per minute
S	susceptible
SAB	Sabouruad chloramphenicol agar
SDD	susceptible-dose-dependent
UAMH	University of Alberta Microfungus Collection and Herbarium
ZD	zone diameter

I. INTRODUCTION

1. Generally about fungi

The Kingdom Fungi contains a diversity of organisms, including macroscopic forms as well as filamentous or yeast-like microscopic structures. Fungi are found throughout nature and play a vital role in the recycling of organic matter. They are often referred to as "saprophytes" or "saprobes", *i.e.* organisms which draw their nourishment from decomposing organic matter. Characteristically, they are eukaryotes, with unicellular or multicellular structures bounded by a rigid cell wall containing chitin. Unlike plants, they do not produce chlorophyll, and obtain nourishment only by absorption of carbon from external sources. They are considered phylogenetically distinct from both plants and animals in their unique synthesis of L-lysine by the L-α-aminoadipic acid pathway. The principal type of sterol present in their cell membranes is ergosterol. Finally, in addition to reproducing asexually, the fungi often possess a form of sexual reproduction [1, 2].

The fungi constitute a very large group of organisms found in virtually every ecological niche. Hawksworth (1991) estimated that, on a worldwide basis, there are about 1.5 million species of fungi [3-5]. To date, however, only about 120 000 species have been described, though the number is steadily increasing [5]. They can be of important in diverse areas such as industry, agriculture and different sciences, including medicine.

2. Medically important fungi

About 200 fungal species are known to cause human infections [6]. Generally speaking, fungal infections of humans are most common in tropical regions of the world. In recent years, however the number of individuals infected with fungi has been increasing drastically in all regions of the world. This is due primarily to the fact that there are more individuals who are predisposed to fungal infections than ever before [3]. The use of antibacterial and immunosuppressive agents contributes to the increase in the number of fungal diseases [7]. Acquired immunodeficiency syndrome (AIDS) patients, for example, are commonly attacked by fungi, as are cancer patients, burn victims and organ transplant patients who have been given immunosuppressive drugs [3, 7]. In some cases, the fungi that attack these individuals are species that are recognized for their abilities to cause infections in humans, but in other cases the species involved are ordinary saprobes not normally regarded as threats to human health [3].

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 [3].

2.1. Yeasts

2.1.1. Morphology of yeasts

Yeasts are fungi that exist as single cells capable of reproducing quickly by budding or fission [3, 5, 6, 8]. Budding involves the production of a small outgrowth (bud) from a parent cell. As the bud is formed, the nucleus of the parent cell divides mitotically, and one daughter nucleus migrates into the bud. The bud 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.) [9]. Budding takes place in the majority of yeasts, but it also occurs in dimorphic fungi, in certain phases of their life or under certain conditions of growth (*e.g. Histoplasma capsulatum*). Fission is a simple splitting of a cell into two daughter cells by constriction and the formation of a cell wall (*Schizosaccharomyces* spp.) [1, 5, 8].

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. Dimorphism is common, for example, in forms that cause diseases in humans. 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* [1, 5, 8].

Some yeasts, such as most *Candida* spp., produce blastoconidia [1, 5, 8, 9]. 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 [1, 5, 8].

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 [2]. They may be terminal or intercalary [1, 5, 8, 10].

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 [11].

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 [6, 9].

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

Candida spp. occur most frequently as human pathogenic yeasts in the phylum Ascomycota, among the Saccharomycetales. 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 [3, 12].

In a study of 52 healthy women (mean age 28 years ± standard deviation 11 years) recruited from the general university community of Iowa City, 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. Half of the test individuals with *Candida* carried the yeast in more than one of the three anatomic sites [13].

In tests on medical students and laboratory workers, the total oral yeast carriage rate determined by a swab method was consistently 25 to 30%, and that of *C. albicans* was 20% [14]. Oral swabs from healthy subjects in various age groups revealed that infants aged 1 week to 18 months have a higher carriage rate (mean 46.3%) than neonates up to 1 week old (mean 17.3%) or children older than 18 months (mean 15%). The carriage rate in adults is higher than that in young children, but lower than that in infants [15]. Among the *Candida* species identified from oral yeast isolates obtained in Europe and North America, *C. albicans*

is the most common species (nearly 70%), followed by *C. tropicalis* (6.7%), *C. glabrata* (6.6%), *C. parapsilosis* (1.9%), *C. krusei* (1.7%), *C. kefyr* (1%) and *C. guilliermondii* (0.4%) [14].

Most studies on the yeast population of the gastrointestinal tract have shown high carriage rates in the stomach and intestines. 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 [16]. These investigators reported the prevalence of yeast as 35% in the oropharynx, 50% in the jejunum, 60% in the ileum and 70% in the colon. *C. albicans* remained the most prevalent species in the gastrointestinal tract (51%), followed by *C. glabrata* (9.1%), *C. parapsilosis* (5.4%), *C. krusei* (2.9%) and *C. tropicalis* (2.3%). *C. guilliermondii* (0.7%) and *C. kefyr* (0.1%) were the least frequent of all *Candida* species [15].

The vaginal yeast carriage rate in females without symptoms is less than 30% [14]. Among vaginal *Candida* cultures, *C. albicans* is the most frequently reported species (69%) from unselected groups of women, followed by *C. glabrata* (11.7%). *C. tropicalis* (5.3%), *C. krusei* (2.6%), *C. guilliermondii* (0.5%) and *C. kefyr* (0.4%) [15].

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 [17].

2.1.3. Pathogenicity of *Candida* spp.

Although healthy individuals show no symptoms of candidiasis, a number of conditions have been reported to favour the predominance of *Candida* organisms within the normal microbial flora. These factors 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 [18]. 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 (a cutaneous infection, a chronic mucocutaneous infection, onychomycosis, an oropharyngeal infection, vulvovaginitis, balanitis, keratitis or conjunctivitis) causing skin, mouth, throat or genital lesions. Individuals of either sex or of any age may develop cutaneous 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 [17].

Candida infection of the genitalia consists of vulvovaginal candidiasis in females and balanitis in males. Vulvovaginal candidiasis occurs most commonly in postpubertal women, who have diabetes mellitus, have been taking systemic antibacterial agents, are in the third trimester of pregnancy, or are sexually active. Although the evidence is inconclusive, oestrogen contraceptive therapy probably predisposes women to the infection [14, 19]. Vaginal discharge, itching, burning, vulvar erythema and oedema are the most common symptoms. Infection is often acute in onset, but may persist for prolonged periods. Although an intestinal reservoir or a sexual partner with *Candida* balanoposthitis has been stated to be the source of recurrent vulvovaginitis, the best current evidence indicates that a relapse results from organisms persisting in the vagina [14, 20].

The same strains may also produce deep candidiasis: localized (oesophagitis, gastrointestinal candidiasis, peritonitis, an urinary tract infection or an intra-abdominal abscess) or haematogenously disseminated infections (candidaemia, chronic disseminated candidiasis, endocarditis, meningitis, endophthalmitis, arthritis or osteomyelitis) [3, 13, 18, 21, 22]. 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 colonisation 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 [23-28]. Colonization of the gastrointestinal tract and other body sites is likely to be the initial step preceding systemic yeast invasion. Severe and prolonged neutropenia following chemotherapy is a major risk factor for systemic fungal infections. However, the proportion of patients without neutropenia or severe immunosuppression who are at risk of invasive fungal infections is higher than usually thought [28]. Carriage of the infecting strain on the hands of health-care workers may also cause nosocomial infections [29]. Five per cent of all nosocomial infections, the majority (86%) of all nosocomial fungal infections and 8-10% of nosocomial bloodstream infections have been shown to be due to *Candida* spp. Thus, *Candida* species are the fourth most frequent nosocomial bloodstream microorganisms [27, 28].

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 [30-32]. Biofilms consist of layers of cells embedded within a matrix of extracellular polymeric material. Detachment of microorganisms from the biofilm can result in septicaemia, which may respond to conventional drug therapy. However, the biofilm itself is R to both host defence mechanisms and antimicrobial agents, so it represents an ongoing source of infection. Consequently, implant-connected infections are difficult to

treat, and the implant must usually be removed [32]. 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 [33].

2.2. Moulds

2.2.1. Morphology of moulds

The identification of filamentous fungi depends on the macroscopic and microscopic examinations of their structures. However, the texture and colour of colonies can be influenced by the culture medium used or by the age of the isolate. Whereas some colonies have a glabrous appearance because they form few aerial hyphae, others are woolly or powdery. Some colonies are pale in colour (whitish, beige, yellowish or pinkish), while others are brightly coloured (green, yellow, cinnamon, pink, red, mauve or violet). Fungi referred to as “dematiaceous” (Dematiaceae, Hyphomycetes) possess a brown, melanin-type pigment in their cell walls; this explains the dull colours of their colonies (grey, brown, olive-brown or black). In some, the pigment is concentrated in their conidia, so that their colonies appear dark on the surface and pale or colourless on the reverse [2].

Moulds are characterized by the development of hyphae, which are seen in the colony characteristic in the laboratory [6, 10]. 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 [2]. Hyphae are developed by apical elongation, which requires a careful balance between cell wall lysis and new cell wall synthesis [6]. It depends on the culture medium, the incubation temperature and the amount of inoculum [2].

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 fruiting bodies called ‘ascomata’, which are often observed as spherical or flask-shaped structures containing asci at first, and then, at maturity, free ascospores. Two types are commonly encountered: the perithecium, which disperses its spores by extruding or shooting them through an apical

opening called an ostiole, and the cleistothecium, which, lacking a specialized opening, splits under pressure and thereby liberates its contents [2].

The overwhelming majority of medically important filamentous fungi are identified by the morphology of their specialized asexual structures (conidia, asexual spores or conidiogenous cells), which are key, even indispensable elements used in the recognition of genera and species. In the Hyphomycetes, a class containing the great majority of medically important filamentous fungi, the conidia are often categorized into those formed through a blastic process or through a thallic process. Blastic conidia are produced in a process that is in essence budding; there is considerable or substantial new wall building in the process of conidial development, and the wall separating the new cell from the mother cell appears only at the time of maturation. By contrast, conidia of thallic ontogeny are the product of hyphal segments, which are already delimited by a septum. These segments then differentiate as specialized conidia [2].

Blastoconidia, which form by a budding process at the apex of a conidiphore, or from another conidium, may be solitary (*Nigrospora* and *Arthrinium*), or in branched chains (*Cladosporium*). Certain types are formed sympodially, on denticles (*Beauveria* and *Sporothrix*) or through pores (*Alternaria* and *Bipolaris*), developing in succession as the conidiophore apex extends and tends to form a zigzag or rosette pattern beneath the extending tip [2].

Phialides are typically bottle-shaped conidiogenous cells, which at their apices produce conidia collecting in mucoid masses (*Fusarium* and *Acremonium*), or remaining attached to each other in unbranched, dry chains (*Aspergillus* and *Penicillium*). Their elongation ceases with the production of the initial conidium [2].

Annelides, like phialides, produce conidia in mucoid masses (*Exophiala* and *Scedosporium*) or in unbranched chains (*Scopulariopsis*). They are distinguished from phialides by continuing to grow in length during the process of conidiation and by the presence of a series of annular rings at their apex. It should be noted that these rings, which are in fact the scars left behind by successive conidia as they break away from the apex, are often difficult to see under the conventional light microscope. In ordinary practice, when it becomes important to distinguish annelides from phialides, one must closely examine the extreme apex of the conidiogenous cell. In particular, for the identification of certain dematiaceous fungi, such as *Phialophora* and *Exophiala*, the annelide tends to be more sharply pointed, whereas the phialide appears truncated [2].

Arthroconidia are conidia of thallic origin, which arise from the transformation of a series of cells along the length of a hypha. They may be contiguous (simple) or intercalated

with separating cells (alternate). At maturity, simple arthroconidia are liberated after the formation of double walls, followed by a process of fission, while alternate arthroconidia are liberated after the lysis of the separating cells. In the latter case, annular frills of remnant wall material can be observed at the ends of the detached conidia [2].

Chlamydospores are inflated, more or less rounded cells, which frequently present with thick walls in older cultures. In contrast with spores and conidia, they lack a dehiscence mechanism allowing them to detach from the fungal thallus when mature. Although they are generally non-specific, they are useful in the diagnosis: they can sometimes serve as an identification marker for certain species, such as the dermatophyte *Trychophyton verrucosum* [2].

Aleurioconidia are terminally or laterally formed conidia liberated by the lysis of the cells which support them. Their truncate bases bearing small annular frills usually characterize them. These frills are remnants of the walls of supporting cells which rived during the process of dehiscence. This type of conidium is characteristic of the dermatophytes and several other fungi of clinical laboratory interest, such as *Chrysosporium*, *Histoplasma* and *Blastomyces*. A process which in some ways appears typically thallic, forms some aleurioconidia; yet, at the same time, many tend to inflate peripherally during formation in a way which resembles blastic conidiation [2].

2.2.2. 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. [34]. Some of them occurred throughout the entire drinking water system, and were thought to constitute a resident fungal flora [34]. *Aspergillus* spp., *Fusarium* spp. and the members of Mucorales are found in the soil, practically universally in the ambient air, therefore colonizing different surfaces by the formation of new branches along the existing hyphae [35-37]. The distribution of spores of different air-borne fungi can vary season by season [38]. Environmental surveys indicate that all humans inhale at least several hundred *A. fumigatus* conidia per day [39, 40]. 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 [41]. 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 [42].

Microsporum gypseum was the most common dermatophyte, and *Rhizopus stolonifer* was the most frequently cultured non-dermatophytic mould in that study [42]. *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 [43, 44]. Members of the *Penicillium* genus are rarely reported as causative agents of infections [45]. Moulds can also occur in human stool flora [46].

A. fumigatus and *S. apiospermum* are well known for their ability to colonize the respiratory tract of cystic fibrosis (CF) patients [47]. The frequency of *S. apiospermum* in bronchial secretions of CF patients appeared very high in comparison with the prevalence of the fungus in environmental studies [47]. According to that study, this species was typically found initially during adolescence. (The mean age of the patients at the date of first isolation was 14.5 years /range 7.8-21 years/, and this fungus was not recovered in young children.) Likewise, *A. fumigatus* frequently emerged during adolescence: the mean age of the patients at the date of first isolation was 12.9 years (range 5-22 years) [47]. However, *S. apiospermum* was usually recovered from the lungs of CF patients who were already colonized with *A. fumigatus*. Like *A. fumigatus*, *S. apiospermum* is able to chronically colonize the airways of CF patients, the total colonization time varying from 10 to 56 months [47]. The specific immune response to *S. apiospermum* of the patients could contribute to the chronic inflammation in the lung, which now appears more important than the microorganisms themselves in the development of tissue damage and pulmonary insufficiency in CF patients. The presence of *S. apiospermum* in the respiratory tract may trigger an inflammatory response that can manifest as an allergic bronchopulmonary disease [47].

Brenier-Pinchart *et al.* investigated the fungal flora in the bronchoscopic fluid of 20 lung transplant recipients [48]. The proportion of patients who displayed filamentous fungi at least once was 90%, and *A. fumigatus* was the most frequently isolated species. Bronchial colonization was detected in 14 patients, invasive bronchial mycosis was diagnosed in 4 others, and there were no cases of invasive pulmonary fungal infection [48].

Fungal surveillance cultures do not reliably predict systemic infection, but they are needed for the prevention of infection in high-risk patients [45, 49]. 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 [45]. Ultrafiltration of ambient air by using high-efficiency-particulate-

arrestor filtration systems for patients at risk greatly reduces the danger of nosocomial filamentous fungal infections in vulnerable hospitalized patients [35].

2.2.3. 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 [45]. 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 [50]. After the inhalation of airborne conidia, macrophages prevent the initiation of infection by phagocytosis and oxidative killing of the spores in immunocompetent hosts [37]. Neutrophils are chemotactically attracted to the hyphae on which they attach and spread. Using their oxidative cytotoxic system, neutrophils damage and kill the fungal elements without accompanying phagocytosis [37].

However, 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 [51]. 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 [51]. *H. capsulatum* has a gene encoding a calcium-binding protein: its presence is essential for virulence [52]. Dermatophytes secrete proteinases, which aid the penetration [53].

Most opportunistic and/or nosocomial infections with filamentous fungi have been associated with nearby construction sites, contaminated airflow ventilation systems, or the care of patients in open wards [35, 45]. 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 [39, 54]. A 4-fold increase in invasive aspergillosis has been observed in the past 12 years, and this infection is now a major cause of death at leukaemia-treatment centres, bone

marrow and solid-organ transplantation units [39, 55]. *A. fumigatus* is responsible for the majority (85-90%) of the different clinical manifestations of severe mould infections [56-58]. 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 [56, 58-68].

3. Antifungal agents and susceptibility testing methods

The development of antifungal agents has lagged behind that of antibacterial agents. 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. Despite these limitations, numerous advances have been made in the development of new antifungal agents and in the understanding of the activities of the existing ones [69].

The polyene antimycotics, such as amphotericin B, nystatin and pimaricin, are all products of *Streptomyces* species. These drugs 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 [69, 70].

Amphotericin B is the main antifungal agent for the life-threatening fungal infections. Its broad spectrum of activity includes most of the medically important dimorphic fungi, including *H. capsulatum, C. immitis* and *B. dermatitidis* [69]. It is useful in the treatment of most opportunistic mycoses caused by fungi such as *Candida* spp., *Cryptococcus neoformans*, *Aspergillus* spp. and the Zygomycetes in immunocompromised patients. Resistance to this agent is rare, but there have been some reports of resistance in non-*albicans Candida* spp. and *Fusarium* spp [70, 71]. Because of its adverse effects (fever, chills, nausea, vomiting, hypotension and renal tubular damage), this drug was incorporated into liposomes in the early 1980s, in an attempt to increase its therapeutic index. Clinical studies confirmed that the liposomal product had significantly less nephrotoxicity than conventional amphotericin B [70, 72, 73].

Nystatin has a broad spectrum of activity against most species of *Candida* and *Aspergillus* [70]. *In vitro* it also inhibits the reproduction of dimorphic fungi, dermatophytes and moulds at different levels [74]. 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 [70, 74]. A lipid formulation of this antifungal agent has been developed for systemic use [75].

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 [69, 70].

When introduced, ketoconazole was the first orally absorbable antifungal azole (bioavailability approximately 75%). Ketoconazole has a range of activity including infections due to *H. capsulatum* and *B. dermatitidis*, for which it is often used in immunocompromised patients. It is also active against mucosal candidiasis and a variety of cutaneous mycoses, including dermatophyte infections and cutaneous candidiasis [69, 70].

The new triazole agents have expanded the possibilities offered by the conventional imidazoles. 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 [70]. 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 [69].

Itraconazole has a broad spectrum of antifungal activity against *Candida* spp., *Aspergillus* spp. and dermatophytes. It accumulates in keratinous tissue and 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 [70].

The widespread use of fluconazole and itraconazole has been accompained by an increase in resistance and by a noticeable shift toward non-*albicans* species with relative resistance to these antifungal agents [76].

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 formulation of voriconazole is well absorbed with a bioavailability of 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 [75].

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 [75]. 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) [75, 77, 78]. The M27-A2 method provides excellent intralaboratory and interlaboratory reproducibility [75]. Qualitative susceptibility results are obtained 24 h sooner by the M44-A method. The correlation of the results obtained with these methods has allowed the establishment of zone interpretative breakpoints for fluconazole and quality control (QC) parameters for fluconazole and voriconazole [75]. 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) [75, 79]. It is applicable for the testing of *Aspergillus* spp., *Fusarium* spp., *P. boydii* and the Zygomycetes [75, 79]. Several multicentre studies have documented the excellent reproducibility of this method, and have shown its promise for the prediction of antifungal efficacy [75]. However, there are moulds with which it is difficult to use, and alternative methods should be considered.

Different commercially available tests have been developed. These alternative methods most often employ a microdilution format, and are read spectrophotometrically or colorimetrically [75]. The results of these tests are sometimes controversial. Moreover, novel breakpoint methods, agar-based methods and flow cytometry have been applied with varying degrees of success [75, 80]. 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 [81].

II. AIMS

1. The identification of yeasts isolated from human samples at a species level is important not only from the view of epidemiology, but also in choosing the antifungal treatment. 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 that occur in human samples, which make possible rapid, exact and cost-effective identification of these isolates in a routine clinical microbiological laboratory.
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, because of their high morbidity and mortality rate, 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.
6. 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, identification and storage 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 [82]. 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. Aerobic and anaerobic blood culture bottles were used in parallel. In some instances, special bottles developed for the detection of fungi were also used. All yeast-positive blood samples were subcultured on CHROMagar Candida (CHR; Becton-Dickinson) for rapid identification of the strains, in parallel with SAB plates. The incubation time was similar as for the other specimens.

If yeast growth was detected on primarily inoculated agar plates during the incubation period, the fungal strain was identified by the conventional methods and/or commercially available tests. The conventional methods included macroscopic and microscopic examinations and biochemical reactions. The morphology, the pigmentation of the colonies and the odour of the culture on SAB helped in the presumptive identification and in the choice of the following identification steps. The colonies of the most frequently isolated *Candida* species (*C. albicans, C. glabrata, C. tropicalis* and *C. krusei*) may have typical colours on CHR [83]. However, the species producing colonies coloured other than green (*C. albicans*) or blue with a lilac pigment (*C. tropicalis*) on this medium need confirmation by some other method. *C. neoformans* produces brown colonies on "*Cryptococcus neoformans* agar" (CNA)

plates (1000 ml CNA containing 45 g sunflower seed, 1 g NaCl, 2.5 g peptone, 2.5 g yeast extract, 10 g glucose and distilled water). LNA is used to recover lipid-dependent *Malassezia* spp. from dermatological samples. Growth at different temperatures, on medium containing 0.1% cycloheximide and/or SAB with cremophore EL may help in the identification.

The germ tube test, growth on rice agar (RA; Difco, Detroit, USA) and an Indian ink preparation have been used for studies of the micromorphology of yeast isolates [84-86]. The carbohydrate assimilation reactions were tested with AUXACOLOR (BIO-RAD, Marnes-La-Coquette, France) supplemented with conventional methods used in routine microbiological laboratory, such as the catalase test, urea and esculine hydrolysis, nitrate reduction and ethanol tolerance [82].

Colonies of moulds grown on SAB were examined macroscopically, similarly as for the yeasts. 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 [84, 87]. 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 [84, 88]. Hydrolysis of urea and *in vitro* hair perforation were carried out according to literature descriptions [88].

Isolates used for further examination were frozen at –70 °C in a CRYOBANK (Mast Diagnostica, Reinfeld, Germany).

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) with the participation of more than 30 countries since June 1997 [89]. In our laboratory, 7089 *Candida* spp. isolates were tested from 2003 to 2005. All strains considered to be pathogenic (causing not only invasive infections), from all body sites (local and systemic) and isolated from patients in different wards, were included. Yeasts considered by the investigator to be colonizers which were not involved in infectious processes were excluded. The isolation and identification of these strains were performed with the routine methods mentioned above.

Susceptibilities for fluconazole and voriconazole were tested by the NCCLS M44-A method [78, 89, 90]. Locally prepared Mueller-Hinton agar medium was supplemented with 2% glucose and 0.5 μg methylene blue per ml. The inoculum was adjusted to match the 0.5 McFarland density standard. The surface of a 90-mm-diameter agar plate was inoculated by

using a swab dipped into the cell suspension. The plates were then incubated for 24-48 h at room temperature or 37 °C, according to the growth conditions for the tested *Candida* species.

Pfizer Inc. supplied 25-μg fluconazole and 1-μg voriconazole disks (manufactured by Becton Dickinson, Sparks, Md.), QC strain *C. albicans* 90028 from the American Type Culture Collection (ATCC; recommended acceptable-performance diameter range of inhibition zone: 28-39 mm for fluconazole and 31-42 mm for voriconazole), and an optional QC strain, *C. parapsilosis* ATCC 22019 (reference inhibition zone range: 22-33 mm for fluconazole and 28-37 mm for voriconazole). Zone diameters were read at 80% growth inhibition, interpreted and recorded by using the electronic image-analysis BIOMIC Plate Reader System. The MIC values reported in this study, were calculated automatically with the BIOMIC System software, according to the data measured for the QC strains weekly [89]. MIC interpretative criteria for fluconazole were S with MIC ≤ 8 μg/ml, susceptible-dose-dependent (SDD) with 16 μg/ml ≤ MIC ≤ 32 μg/ml, and R with 64 μg/ml ≤ MIC [77]. The corresponding zone interpretative criteria were S with zone diameter (ZD) ≥ 19 mm, SDD with 15 mm ≤ ZD ≤ 18 mm, and R with ZD ≤ 14 mm [89, 90]. Although MIC breakpoints have not yet been established for voriconazole, a single preliminary interpretative category of susceptibility exists: MIC ≤ 1 μg/ml, which is supported by pharmacokinetic and pharmacodynamic parameters [90].

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 [91, 92]. 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. For 10 mould isolates, the test was carried out by using two different turbidity suspensions (0.5 and 3 McFarland) in parallel. The drug concentrations of the Etest strips were 0.016-256 μg/ml for fluconazole and 0.002-32 μg/ml for itraconazole, ketoconazole and amphotericin B. The incubation time was 24 to 48 or 72 h at 37 °C (yeasts) or 30 °C (moulds), the time depending on the growth characteristics of the species. The MICs for amphotericin B were taken as the drug concentrations causing 100% inhibition. MICs for azoles were read at the visually selected endpoint of 80% inhibition of growth. Interpretative susceptibility criteria for these antifungal agents were used as published by the NCCLS and in the literature [77, 82].

The susceptibilities of 10 *Trichoderma* strains were evaluated for fluconazole and amphotericin B by the agar dilution method [93]. Stock solutions of 2 g/l were prepared from

amphotericin B (Bristol-Myers Squibb AG, Baar, Switzerland) and fluconazole (Pfizer, Amboise, France) in sterile distilled water. Appropriate quantities of the stock solutions were added to 20 ml molten and cooled RPMI 1640 agar supplemented with 2% glucose to reach final drug concentrations of 4-512 μg/ml for fluconazole and 0.25-32 μg/ml for amphotericin B, corresponding to the Etest concentrations. Three-mm-diameter discs were cut from the edges of young colonies of the culture. One disc was inoculated onto each plate, and the plates were then incubated at 30 °C for 48 h. 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 first step was the hypotonic lysis of the erythrocytes: 500 μl blood sample was treated with 300 μl erythrocyte lysis buffer diluted in 700 μl distilled water [94]. It was mixed and incubated for 5 min at room temperature. The fungal cells were precipitated by centrifugation for 10 min at 8000 rotations per minute (rpm). The supernatant was eliminated, and the sediment was washed once with 500 μl distilled water. After mixing, the material was centrifuged again as previously described. The sediment was resuspended in 200 μl digestion buffer (containing 50 mM Tris buffer solution, 1 mM EDTA and 28 mM β-mercaptoethanol) and 20 μl 10% *Helix pomatia* gastric juice to generate spheroplasts. After incubation for 10 min at 35 °C with shaking (500 rpm), samples were centrifuged at 8000 rpm for 2 min. The supernatant was discarded, and 200 μl ATL buffer and 20 μl proteinase K were then pipetted onto the sediment. This step and the further processing were carried out according to the manufacturer's instructions for use of the QIAamp Tissue Kit (QIAGEN GmbH, Hilden, Germany).

The primers used for universal fungal amplification were internal transcribed spacer (ITS)4 region (TCC TCC gCT TAT TgA TAT gC) as reverse, ITS1 (TCC gTA ggT gAA CCT gCg g), ITS3 (gCA TCg ATg AAg AAC gCA gC) and ITS86 (gTg AAT CAT CgA ATC TTT gAA C) as forward. The size of amplified PCR products varied between 300 and 500 base pairs (bp), depending on the fungal strains and the forward primer applied.

The PCR was performed in disposable capillaries with a LightCycler 1.5 (Roche Diagnostics Corporation, Indianapolis, Indiana). The reaction volume was 10 μl containing 1 μl sample desoxyribonucleic acid (DNA) prepared previously, 1 μM each of the primers, 5

µl reaction buffer (SYBR®Green Taq ReadyMix, Sigma, St. Louis, Missouri, U.S.A.) and 0.3 µl 25 mM $MgCl_2$ stock solution. The PCR conditions were as follows: initial denaturation at 95 °C for 60 s, followed by 35 cycles of denaturation (95 °C for 1 s, 20 °C/s), annealing (55 °C for 15 s, 20 °C/s), and extension (72 °C for 25 s, 2 °C/s). The melting curve analysis consisted of 1 cycle at 72 °C for 20 s, after which the temperature was increased to 95 °C at 0.1 °C/s continuously. The fluorescence signal (F) was monitored continuously during the temperature increase, and was then plotted against temperature (T). These curves were transformed to derivative melting curves: (-dF/dT) *vs* T.

PCR amplification was confirmed by the visualization of PCR products by agarose gel electrophoresis. The agarose gel consisted of 2% Top Vision agar (Fermentas, Burlington, Canada) dissolved in Tris-borate-EDTA buffer (0.1 M Tris, 0.09 M boric acid, 0.001 M EDTA; pH 8.4). 10 µl amplicon was combined with 2 µl Loading Dye (Fermentas), and then added to the agarose gel well. The GeneRuler™ 100 bp DNA Ladder (Fermentas) was used for the exact fragment analysis. Electrophoresis was conducted at 120 V for 30 to 45 min. The gel was examined with a UV transilluminator, and analysed with the Kodak EDAS 290 system (Eastman Kodak Company, Rochester, New York, U.S.A.).

IV. RESULTS

1. The schemes for identification of human pathogenic yeasts

Fungal infections are significant causes of morbidity and mortality in immunocompromised patients. The incidence of infections caused by yeasts is also increasing among immunocompetent patients. The exact identification of pathogenic yeasts is important not only from an epidemiologic aspect, but also in the choice of appropriate antifungal agent. Microbiological laboratories in Hungary occasionally identify yeast isolates at a species level. If this is the case, the spectrum of the species is limited, and the identification of the species is based only on the results of commercially available (biochemical) tests. However, these results should always be confirmed with morphological examinations. The aim of this work was to devise comprehensive identification schemes for human pathogenic yeasts isolated with low or high incidence in a routine microbiological laboratory.

When yeast is cultured from a clinical specimen, the first step is the macroscopic examination of the colonies on SAB plates. If the colonies are buttery, whitish to cream-coloured, smooth and soft, germ tube tests are carried out. If germ tube production can be detected in the serum, the strain can be identified as *C. albicans*. If it is not able to produce a germ tube, the isolate should be inoculated onto RA and CHR plates in parallel. After a 24-72-h incubation, the micromorphology and the colour of the colony are examined on RA and CHR, respectively. *C. albicans* has green colonies on CHR; it produces pseudohyphae with blastoconidia in a dense, grape-like arrangement and terminal chlamydospores on RA. If the strain produces blue or greyish-purple colonies on CHR, and alilac pigment is visible in this agar medium near the colonies, and if on RA, blastoconidia are formed singly or in very small groups along the abundantly produced pseudohyphae, the strain is identified as *C. tropicalis*.

If the colour of the colony on CHR differs from blue or green, the absence or the presence of pseudohypha production should be checked on RA. *C. glabrata* forms greyish, soft, glossy colonies on SAB, and purple, greyish-purple or white colonies on CHR. On RA, it does not produce pseudohyphae, the budding is unipolar, and the cells are small, ellipsoidal, and typically arranged in dense groups. *C. inconspicua* does not produce pseudohyphae on RA either: only a mass of elongated cells can be seen by microscope. It produces white, flat colonies with a typical sweetish odour on SAB and CHR plates. The growth of the colonies of *S. cerevisiae* is restricted on SAB; they display peaks, and are cream-coloured. The budding cells are spherical, with multilateral bud formation; ascospores may be seen inside the cells. Some pseudohyphae may be present. It produces dark-purple colonies on CHR. *M. pachydermatis* is the only *Malassezia* species, which produces cream-coloured, convex, soft

colonies with a fully or slightly lobed margin on SAB. This genus has typical micromorphology with paired cells. In the case of *M. pachydermatis*, the buds are as wide as the mother cells. If a yeast isolate is obtained which produces whitish or cream-coloured colonies on SAB, germ tube and pseudohypha production is not detectable, there are no green or blue colonies on CHR, and it is not identified as one or the other of the species mentioned above, a urease test should be carried out. If it is urease-negative, the assimilation of galactose and raffinose may help to identify *C. famata*, *C. pulcherrima* or *C. utilis*. *C. famata* can also assimilate galactose and raffinose, *C. pulcherrima* can utilize only galactose, while *C. utilis* utilizes only raffinose. If the urease test is positive, the isolate may be a member of the *Cryptococus* genus. Indian ink staining can confirm this, but not every *Cryptococcus* species has capsules. When cultured on CNA at 37 °C for 24-72 h, *C. neoformans* produces brown-pigmented colonies. If *C. albidus* is grown on CNA plates under similar circumstances, it does not produce pigmented colonies, but the growth of this species at this temperature varies. Further differentiation of *Cryptococcus* species not growing on CNA plates at 37 °C is based on their biochemical reactions summarized in Table 1.

If pseudohyphae are present on RA, if the colonies on SAB are smooth, whitish to cream-coloured, soft, and the if colour on CHR is other than blue or green, the species can be identified via its carbohydrate-assimilation profile and its ability to grow on medium containing 0.1% cycloheximide, as indicated in Table 2.

The scheme for the identification of yeast isolates with buttery, whitish to cream-coloured, smooth, soft colonies on SAB is summarized in Figure 1.

Various human pathogenic yeast species produce wrinkled, cerebriform, dry, farinose, deeply fissured or flat colonies on SAB after a 24-h incubation or later. The first step in the identification of these species may be to check the existence or lack of arthroconidia. This can be achieved by microscopic examination of the cell suspension or the growth on RA. If arthroconidia are visible, the urease test can help in the differentiation: *Trichosporon* spp. are urease-positive, while *Geotrichum* spp. are not able to hydrolyse urea. The identification of *Trichosporon* spp. at a species level is possible by biochemical methods and morphological examinations (Table 3). Their colonies on SAB are initially yeast-like with a deeply fissured marginal zone, but later become cerebriform or farionose. Among *Geotrichum* spp., *G. candidum* assimilates xylose, but not cellobiose, *G. clavatum* assimilates cellobiose, but not xylose, and *G. capitatum* assimilates neither of them. Members of the *Geotrichum* spp. produce farinose, filamentous fungus-like colonies on SAB.

Other species which do not produces arthroconidia are cultured onto CHR and RA. The micro- and macromorphology of *C. tropicalis* and *C. inconspicua* on these media are

described above. *C. parapsilosis* can be identified by its micromorphology on RA (blastoconidia singly or in small clusters along the curved pseudohyphae, occasionally with large hyphal elements called “giant cells”) and its purplish-white colonies on CHR, and it does not assimilate cellobiose. Other supplementary characteristics of this species are listed in Table 2. *C. krusei* produces flat, dry colonies on SAB, and purplish-white or pinkish, dry colonies on CHR. It forms pseudohyphae with elongated blastoconidia, creating a tree-like appearance on RA. These characteristics are similar in the cases of *C. lipolytica* and *C. lambica*, but neither of these latter two species grows at 42 °C, whereas *C. krusei* does. *C. lambica* is a urease-negative, *C. lipolytica* is a urease-positive species.

For further identification of arthroconidia-negative isolates other than *C. parapsilosis*, *C. tropicalis* and *C. inconspicua*, the hydrolysis of urea, the assimilation of inositol and nitrate reduction should be tested. A urease-positive, inositol-positive and nitrate-negative isolate is *Cryptococcus humicola*. Urease-positive, inositol-negative and nitrate-negative isolates can be *C. lipolytica* or *C. krusei*. The differentiation between these two species is based on the growth at 42 °C. Urease-negative, inositol-positive and nitrate-negative isolates are *C. chiropterorum* or *C. ciferrii*. *C. chiropterorum* does not grow in the presence of ethanol, whereas *C. ciferrii* does. There is one urease-negative, inositol-negative and nitrate-positive species: *C. catenulata*. *C. krusei* may be urease-, nitrate- and inositol-negative, like *C. rugosa*, *C. lambica*, *C. viswanathii* and *C. zeylanoides*. *C. viswanathii* and *C. zeylanoides* are able to grow on media containing 0.1% cycloheximide; the former is maltose-positive, while the latter is maltose-negative. Glucose fermentation, pellicle production in broth and growth at 42 °C help in the differentiation of cycloheximide-negative species (Table 4).

The identification scheme for the yeast isolates with wrinkled, cerebriform, dry, farinose, deeply fissured or flat colonies on SAB is summarized in Figure 2.

Malassezia species produce cream-coloured or yellowish, slightly wrinkled colonies with or lobed or entire margin on SAB and/or LNA at 30 °C. If a *Malassezia* spp. isolate is able to grow on SAB, it is identified as *M. pachydermatis* (Figure 1). Other *Malassezia* species growing only on LNA should be cultured and incubated at 40 °C. Among isolates not growing at this temperature, *M. restricta* is the only catalase-negative species. Esculine hydrolysis should be tested in the event of catalase-positive species: *M. globosa* is esculine-negative, while *M. obtusa* is positive.

Malassezia spp. growing at 40 °C on LNA are examined microscopically on RA. If the buds are narrower than the mother cells, the result of the identification process is *M. sympodialis*. When the buds are as wide as the mother cells, culturing on SAB with

cremophore EL helps to decide between *M. furfur* and *M. slooffiae*: the former species grows on this medium, while *M. slooffiae* does not (Figure 3).

Yeasts producing red-, orange- or salmon-pigmented colonies on SAB may be *Sporobolomyces salmonicolor*, *Rhodotorula* spp. or *Cryptococcus macerans*. These fungi can be identified by their micromorphology on RA, their ability to assimilate nitrate and/or raffinose and their growth at 30 °C on SAB. The identification flow chart for these fungi is depicted in 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 (Table 5). 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 (Table 5).

2. Computer-assisted antifungal susceptibility testing of *Candida* spp. isolates (Paper I)

The ARTEMIS Disk Study is organized for the evaluation of fluconazole and voriconazole susceptibility testing results obtained by the disk diffusion method for human pathogenic yeasts. Our laboratory joined in this study in 1999. Both examined antifungal agents have been tested simultaneously since 2001. The data collected in our laboratory from 2001 to 2003, together with those originating from the other Hungarian participants, are summarized in Paper I. The aim of this work was an evaluation of the antifungal susceptibility testing results obtained in our laboratory for *Candida* spp. from 2003 to 2005.

During the 3-year period, 7089 *Candida* spp. strains were tested within the frame of the study. The total numbers of isolates were similar in 2004 and 2005, but the number in 2003 was higher (Table 6). 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, than that of *C. tropicalis* 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% (Table 6).

The fluconazole susceptibility testing results are summarized in Table 7. The majority 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 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. 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 in 2005.

Only a single preliminary interpretative category of susceptibility exists for voriconazole. The efficacy of this new antifungal agent 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 8. 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 $\leq$ 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 8).

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

The bloodstream infections caused by yeasts have high morbidity and mortality rates, mainly among immunosuppressed and trauma patients. The most frequent pathogens are members of the *Candida* genus. Besides their exact identification at a species level, the antifungal susceptibility testing is important from the aspect of curing the patient. The aim of this work was to establish the occurrence of different *Candida* species originating from blood cultures received from different clinical wards of the University Hospital in Szeged, Hungary, during a 10-year period. The susceptibilities of these isolates to antifungal agents were also examined.

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%). The species distribution by years is summarized in Table 9. In some cases, the same species was isolated from more than one blood culture bottle from the same patient. There were 4 patients during this period (2001-2005), 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 (Table 10). 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).

Before 1997, the susceptibilities of *Candida* spp. isolated from blood cultures were not determined routinely. The recommendation for the antifungal treatment was based on the species properties (*e.g.* the fluconazole resistance of *C. krusei* and *C. glabrata*). After 1997, isolates were tested for their susceptibilities to fluconazole, itraconazole, ketoconazole and amphotericin B. Among the *C. albicans* isolates, 92.6% and 82.8% were S to fluconazole and itraconazole, respectively (Table 11). 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 than to itraconazole. 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 (Table 11).

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

Invasive fungal infection is a major cause of morbidity and mortality in immunocompromised patients. The early initiation of antifungal therapy is of paramount importance if the high mortality rates associated with fungaemia are to be reduced. The current gold standard for the detection of systemic infection is blood culturing, but this is believed to lack sensitivity, has been shown to be positive in less than 50% of patients with chronic disseminated candidiasis, and is rarely positive for patients with invasive aspergillosis. It is essential to overcome the limitations of the traditional culture-based fungal detection methods, and replace them with a rapid and sensitive molecular genetic procedure. A real-time PCR method appears suitable for this purpose.

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 personal and clinical data, and the microbiological results on the patients are summarized in Table 12. The first group of patients (1-13) 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 (Table 12).

The third group in Table 12 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 history of a 45-year old man (patient 17) with acute pancreatitis is analysed chronologically (Figure 5). Before his operation, he was in a surgical department on 9 and 10 September 2004. Blood samples collected and cultured on 9 September were negative for fungi. However, *C. glabrata* was cultured from the intraoperative sample (originating from a retroperitoneal abscess) collected on 10 September. The first blood sample was collected for PCR in the surgical ICU on 15 September, after *C. glabrata* and *C. inconspicua* were isolated from a bile sample (on 12 September). Fluconazole was administered empirically from 11 to 14 September. Blood samples collected for culturing during his stay in the ICU (from 12 to 21

September) were negative for fungi and bacteria; other samples (from peritoneal drainage) were positive only for bacteria (*Proteus morganii* and *Pseudomonas aeruginosa*), with the exception of the bile specimen mentioned above. Blood samples for PCR were collected almost daily from 15 to 22 September. Of these 7 samples, one was positive with PCR for fungi (*Candida* spp.), but antifungal therapy was not administered at that time. The status of the patient allowed his transfer to the surgical department (21 September), because he no longer needed intensive care. Two blood samples were sent to the laboratory on 26 and 27 September, and one of them was positive by PCR. After this, no further PCR samples were collected. *C. glabrata* was cultured from 2 ascites samples collected on 30 September. No other specimens were collected for microbial culturing from 1 to 14 October, and antifungal treatment was not administered. Specimens (other than blood samples for culturing) collected on and after 14 October (during his further hospitalization) were positive for bacteria (*Enterobacter aerogenes*, *E. cloaceae*, *Enterococcus faecalis*, *Citrobacter freundii* and *Staphylococcus epidermidis*), but not for fungi. However, blood samples collected on 14 October were positive (after a 19-h incubation) for *C. glabrata* and *E. aerogenes*, while the following one, collected on 19 October, was positive (after a 170-h incubation) only for *C. glabrata*. His antifungal treatment (amphotericin B) was started only from 21 October, and was continued until 8 November. He was discharged home on 28 November.

The last group contains 5 patients (18-22; Table 12) 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. Patient 22 was an immunocompromised woman with pneumonia caused by cytomegalovirus. Her *Aspergillus* spp. antibody (Ig class not known) was also positive (data not shown).

5. Clinical importance of environmental filamentous fungi in human infections (Papers III, IV, V and VI)

The number of disseminated infections caused by filamentous fungi is lower than that caused by yeasts. However, the increasing number of susceptible hosts (organ or bone marrow transplant patients) and the use of immunosuppressive agents and antimicrobial prophylactic strategies have probably contributed to the changing epidemiology of mycoses. Most infections caused by moulds originate from the environment by inhalation through the respiratory tract, trauma or contamination. The most frequently isolated moulds are *Aspergillus* species, but the importance of other filamentous fungi, such as the *Fusarium* spp. and *Trichoderma* spp., has also been increasing.

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 (fast-growing white colonies with abundant aerial mycelium, delicate, slender, falcate, 3-5 septate macroconidia, and microconidia abundantly produced in chains). 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 (Papers IV, VI and VII)

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: mucin from the nose, washing fluid from the paranasal sinuses or nose, specimens from the ears or thoracic fluid. 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 (Table 13). 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 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. The MICs obtained after incubation for 48 or 72 h are listed in Table 14. For fluconazole, no differences were observed with the two inocula: all the strains were fully R, with MIC > 256 μg/ml (data not presented). 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 (Table 14).

The MICs of fluconazole and amphotericin B obtained for 10 *Trichoderma* strains by the Etest and the agar dilution method were compared (Table 15). 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 identification of human pathogenic yeasts is a basic requirement in routine clinical microbiological laboratories because of their increasing incidence. 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 [83, 84, 95]. *C. tropicalis* was reported to form germ tubes, but this ability was rapidly lost during repeated subculturing [95]. 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.

C. dubliniensis is an emerging pathogen capable of causing oropharyngeal, vaginal and bloodstream infections [96]. Its phenotypic characteristics 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 [96, 97]. The use of this in routine microbiological laboratory practice would be expensive. Nevertheless, there are no consequences of the misidentification of *C. albicans* and *C. dubliniensis* for antifungal therapy [96-98]. The rate of isolation of *C. dubliniensis* is also small (0.2% in Kuwait, 0.17% in Turkey and 1.3% in Belgium) [99-101].

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* [83]. 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. In our laboratory, the results of other identification methods are always compared with this micromorphological picture on RA (except for the germ tube-producing *C. albicans*). 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.

The time and/or the cost of the exact identification may be decreased by acquisition of mycological practice with the frequently isolated yeast species (*C. albicans*, *C. glabrata*, *C. krusei*, *C. inconspicua*, *C. kefyr*, *C. tropicalis*, *C. parapsilosis*, *G. candidum* and *S. cerevisiae*).

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 (Table 5). 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. These identification methods were used for the yeast isolates originating from the bloodstream infections mentioned later.

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

The BIOMIC Plate Reader System is suitable for collecting valuable epidemiological data and susceptibility testing results. These data are comparable in a global study, and also allow the comparison of data generated in a country or in a laboratory year by year. The changes in the epidemiological data and in the trends of the antifungal susceptibility or resistance of pathogenic yeasts can be followed.

The numbers of tested isolates were smaller in 2004 and 2005 than in 2003 because different technical disorders appeared, which influenced the quantity, but not the quality of the measurements (Table 6). The relative quantities of tested isolates correlated with the number of strains isolated in our laboratory. 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 [89]. The rate of *C. glabrata* was 11% of the total number of *Candida* spp. strains tested in Hungary in 2004 (data not presented). 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 (Table 6). 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 [89].

The fluconazole susceptibility results obtained for *C. albicans* isolates in our laboratory were very similar to the Hungarian ones in 2003 (Table 7). 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 (Table 7).

According to the literature data, voriconazole exhibited markedly enhanced *in vitro* activity against most of the yeast isolates originating from different human specimens [89]. 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 8). 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 (Table 9). 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. Because of the development of the identification methods used in the laboratory, no *Candida* spp. have been isolated since 2002 (Table 9).

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 [102-108]. Whereas, the rates of isolation of *C. glabrata* (15.1%) and *C. parapsilosis* (17.8%) were similar in a French report, these values were 13.9% and 8% respectively, according to our data [108]. 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%) [104].

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 (Table 10). 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 (Table 10).

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 [104, 105, 109]. The rates for *C. albicans* strains found to be SDD or R to fluconazole were similar (Table 11). 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% [104]. 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 [110]. 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 (Table 11).

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

Fungal bloodstream infections have high morbidity and mortality rates, mainly among immunosuppressed patients [111]. Several microbiological methods are available for their detection: culturing, antigen detection and molecular genetic methods. The time is an important factor in the laboratory diagnosis, and may influence the outcome of the infection. Molecular genetic methods are sensitive, and allow rapid microbiological diagnosis. The ITS region is now perhaps the most widely sequenced DNA region in fungi. It has typically been most useful for the detection of fungi, for taxonomy at a species level and even within species on the basis of their molecular genetic properties.

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 (Table 12). 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) [112].

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. Fluconazole treatment was started, when *C. glabrata* was cultured from his intraoperative specimen. However, fluconazole is not recommended for the treatment of infections caused by *C. glabrata* or *C. inconspicua*. Of the 9 blood samples taken between 15 and 27 September for the PCR detection of fungi, 2 were positive (Figure 5). 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 (Table 12). 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 [113].

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

Members of the *Fusarium* genus occur in the soil all over the world. Certain species are plant pathogens, while others are soil saprophytes. 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 [114-116]. However, fusarial keratitis is rare in European countries with temperate climates: 4 cases have been reported from Paris, France during an 8-year period [117]. The species reported most often was *F. solani* [118].

Corneal trauma is the most common predisposing factor. The injuries are caused by various traumatizing agents, including plant material (paddy, a tree branch, thorn, hay, sugar

cane, grass, corn stalks, onions, ground nuts, kernel and palm leaf), animal matter (a cow's tail, cow dung, insects, cat scratches and hen peck), 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 (*e.g.* chronic dacryocystitis), 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. Out of 159 cases of keratitis caused by *Fusarium* spp. in Florida, 10 patients progressed to endophthalmitis [119].

Trichoderma species are also potential opportunistic pathogenic filamentous fungi. They have different virulence factors, which may play important roles in causing human infections. Growth at elevated temperature (37 °C) and the tolerance of neutral pH are necessary for the colonization of the human environment. All of the examined clinical strains were able to grow under the circumstances mentioned above, and this ability can therefore promote their growth as facultative human pathogens. Other ecophysiological studies of *Trichoderma* genus have revealed that species belonging in the Longibrachiatum section have higher optimum growth temperatures [120]. 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) [121].

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

Most of the infections caused by moulds originate from the environment. These fungi pass into the human body through breathing, food or contamination. The susceptibility testing of filamentous fungal isolates is important when the risk of invasive infection exists. There have been previous reports on comparisons of antifungal susceptibility testing methods for moulds, and a good correlation was observed between the Etest and broth microdilution methods [56, 122]. In this study, most strains were R to fluconazole by the Etest, while ketoconazole, itraconazole and amphotericin B were more effective (Table 13). For most strains, the MICs were not influenced significantly by the turbidity of the spore suspension (Table 14). 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 (in accordance with the manufacturer's instructions). 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 (Table 15). 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 [56, 122].

VI. CONCLUSIONS

1. We have developed identification schemes for yeasts isolated more or less frequently from human specimens: both *Candida* spp. and *Malassezia* species are involved. 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. These examinations are cost-effective, and thus (supplemented with practice in clinical mycology) the exact identification of yeasts cultured from human speciemens can be available in many routine microbiological laboratories. The importance of these schemes lies not only in the choice of antifungal therapy, but also in the epidemiology.
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. This method is suitable for the collection of many epidemiologic and susceptibility testing results. These data are comparable with others not only from Hungary, but also from other participating countries, and also with the summarized global results. Thus, trends or changes in the antifungal susceptibilities may be followed, 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. The most frequent species causing bloodstream infection during the evaluated 10-year period was *C. albicans*, but the number of episodes caused by *C. glabrata* was the same because of an epidemic in 2005. Our aetiological and antifungal susceptibility data collected during a 10-year period are comparable with others originating from other countries. The monitoring of bloodstream infections caused by fungi is necessary because of their high morbidity and mortality rates, and the gold standard for their detection is the blood culture system.
4. We introduced and optimized a DNA preparation method and a real-time PCR method, suitable for the detection of fungi in blood samples. The primers used in this reaction are localized on the ribosomal RNA encoding the DNA genes of the fungi. These are multiple-copy and conserved genes in the fungal genome. Thus, they are

suitable for the examination of the presence or absence of fungi in blood samples, for the detection of fungaemia and for the verification of results obtained by culturing. Results can be obtained with this DNA preparation method and the real-time PCR method within 3 h, whereas the culturing needs at least 24 h.

5. The epidemiology of environmental filamentous fungi (*Fusarium* spp. and *Trichoderma* spp.) isolated from human samples was examined. For the first time in Hungary, we published a case of fungal keratitis caused by *F. verticillioides*. We examined 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. The effects of temperature and pH on the growth of isolates originating from human samples were examined as potential virulence factors. 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 (human or environmental) origins were compared in this study. The results obtained under different testing circumstances (incubation time and turbidity of spore suspension) and different susceptibility testing methods (Etest, broth micro- and macrodilution and agar dilution methods) were evaluated. For most strains, the MICs were not influenced significantly by the turbidity of the spore suspension. If the higher inoculum was used for some slow-growing isolates, the MICs could be read 1 day earlier. 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.

VII. ACKNOWLEDGEMENTS

I wish to express my special thanks to all those without whom this work would never have been completed:

I am deeply grateful to my supervisor, **Professor Dr. Elisabeth Nagy**, whose constant scientific guidance, encouragement and support over the past years have been invaluable.

I express my grateful thanks to **Dr. László Kredics** and **Dr. Zsuzsanna Antal** for their wide-ranging cooperation.

I wish to thank **Professor Dr. Csaba Vágvölgyi** and **Dr. János Varga** for their great help in the identification of filamentous fungi.

I am especially grateful to **Dr. Edit Hajdú** and the **staff of the clinics** for their tremendous help in data collection.

I am also especially grateful to **Dr. Ferenc Somogyvári** for sharing with me his vast knowledge on the real-time PCR method.

My warmest thanks are also due to **Edit Komjáti**, **Szilvia Batki** and **all the members of the staff of the Department of Clinical Microbiology**, whose indispensable technical assistance has contributed so much to this work.

I wish to thank **Gabriella Vad** for the highly efficient office work.

And last, but not least, I would like to thank **my parents** for the love and every other type of support that I have received during these years.

VIII. REFERENCES

1. Characteristics of fungi. *In:* Alexopoulos, C. J., Mims, C. W. and Blackwell, M.: Introductory mycology. Fourth edition. John Wiley & Sons, New York, U. S. A., 1996; 26-60.
2. Criteria of identification. *In:* St-Germain, G. and Summerbell, R.: Identifying filamentous fungi. A clinical laboratory handbook. Star Publishing Company, Belmont, California, U. S. A., 1996; 3-9.
3. Kingdom Fungi. Introduction to fungi and their significance to humans. *In:* Alexopoulos, C. J., Mims, C. W. and Blackwell, M.: Introductory mycology. Fourth edition. John Wiley & Sons, New York, U. S. A., 1996; 1-25.
4. Hawksworth, D. L.: The fungal dimension of biodiversity: Magnitude, significance and conservation. *Mycol Res* 1991; **95:** 641-655.
5. A gombák általános jellemzése, helyük, szerepük az élővilágban. *In:* Varga, J., Kucsera, J., Kevei, F. and Vágvölgyi, Cs.: Fejezetek a mikológiából. JATEPress, Szeged, Hungary, 1999; 5-35.
6. McGinnis, M. R.: Introduction to mycology. *In:* Medical microbiology. Edited by Baron S. Third edition. Churchill Livingstone Inc., 1991; 921-933.
7. Walsh, T. J. and Dixon, D. M.: Spectrum of mycoses. *In:* Medical microbiology. Edited by Baron S. Third edition. Churchill Livingstone Inc., 1991; 951-957.
8. Gomba sejt, gombatest (thallus) szerveződése, vegetatív és ivaros képleteik. *In:* Kevei, F., Kucsera, J.: Mikrobiológia I. JATEPress, Szeged, Hungary, 1998; 234-258.
9. Yeasts and yeastlike organisms. *In:* Larone, D. H.: Medically important fungi. A guide to identification. Second edition. Elsevier Science Publishing Co., Inc. Amsterdam, The Netherlands, 1987; 53-75.
10. Glossary. *In:* Larone, D. H.: Medically important fungi. A guide to identification. Second edition. Elsevier Science Publishing Co., Inc. Amsterdam, The Netherlands, 1987; 213-220.
11. Yeasts and yeast-like fungi: explanatory chapter, key to the genera and descriptions. *In:* de Hoog, G. S., Guarro, J., Gené. J. and Figueras, M. J.: Atlas of clinical fungi. Second edition, Utrecht, Netherlands, 2000; 125-236.
12. Hopfer, R. L.: Mycology of *Candida* infections. *In:* Candidasis. Edited by Bodey, G. P. and Fainstein, V. Raven Press Books, Ltd., New York, U. S. A., 1985; 1-12.

13. Soll, D. R., Galask, R., Schmid, J., Hanna, C., Mac, K. and Morrow, B.: Genetic dissimilarity of commensal strains of *Candida* spp. carried in different anatomical locations of the same healthy women. *J Clin Microbiol* 1991; **29:** 1702-1710.
14. Candidiasis. *In:* Kwon, K. J. and Bennett, J. E.: Medical mycology. Lea and Febiger, Philadelphia, Pennsylvania, U. S. A., 1992; 280-336.
15. Ecology of *Candida* and epidemiology of candidosis. *In:* Odds, F. C.: *Candida* and Candidosis. Second Edition. London, Bailliere Tindall, United Kingdom, 1988; 68-90.
16. Cohen, R. Roth, F. J., Delgado, E., Ahearn, D. G. and Kalser, M. H.: Fungal flora of the normal human small and large intestine. *N Engl J Med* 1969; **280:** 638-641.
17. Rosen, T.: Cutaneous candidiasis. *In:* Candidasis. Edited by Bodey, G. P. and Fainstein, V. Raven Press Books, Ltd., New York, U. S. A., 1985; 227-240.
18. Smith, C. B.: Candidiasis: Pathogenesis, host resistance and predisposing factors. *In:* Candidasis. Edited by Bodey, G. P. and Fainstein, V. Raven Press Books, Ltd., New York, U. S. A., 1985; 53-70.
19. Gentry, L. O. and Price, M. F.: Urinary and genital *Candida* infections. *In:* Candidasis. Edited by Bodey, G. P. and Fainstein, V. Raven Press Books, Ltd., New York, U. S. A., 1985; 169-179.
20. O'Connor, M. I. and Sobel, J. D.: Epidemiology of recurrent vulvovaginal candidiasis: identification and strain differentiation of *Candida albicans*. *J Infect Dis* 1986; **154:** 358-363.
21. Az orvosi mikológia néhány vonatkozása. *In:* Varga, J., Kucsera, J., Kevei, F. and Vágvölgyi, Cs.: Fejezetek a mikológiából. JATEPress, Szeged, Hungary, 1999; 247-285.
22. Guide to interpretation of direct microscopic examination of clinical specimens. *In:* Larone, D. H.: Medically important fungi. A guide to identification. Second edition. Elsevier Science Publishing Co., Inc. Amsterdam, The Netherlands, 1987; 8-15.
23. Beck-Sagué, C. and Jarvis W. R.: Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980-1990. National Nosocomial Infections Surveillance System. *J Infect Dis* 1993; **167:** 1247-1251.
24. Berenguer, J., Fernández-Baca, V., Sánchez, R. and Bouza, E.: *In vitro* activity of amphotericin B, flucytosine and fluconazole against yeasts causing bloodstream infections. *Eur J Clin Microbiol Infect Dis* 1995; **14:** 362-365.
25. Martin-Mazuelos, E., Gutiérrez, M. J., Aller, A. I., Bernal, S., Martinez, M. A., Montero, O. and Quindos, G.: A comparative evaluation of Etest and broth microdilution methods for fluconazole and itraconazole susceptibility testing of *Candida* spp. *J Antimicrob Chemother* 1999; **43:** 477-481.

26. Nagy, E.: Changing epidemiology of systemic fungal infections and the possibilities of laboratory diagnostics. *Acta Microbiol Immunol Hung* 1999; **46:** 227-231.
27. Singh, N.: Changing spectrum of invasive candidiasis and its therapeutic implications. *Clin Microbiol Infect* 2001; **7 (Suppl. 2):** 1-7.
28. Muñoz, P., Burillo, A. and Bouza, E.: Environmental surveillance and other control measures in the prevention of nosocomial fungal infections. *Clin Microbiol Infect* 2001; **7 (Suppl. 2):** 38-45.
29. Levin, A. S., Costa, S. F., Mussi, N. S., Basso, M., Sinto, S. I., Machado, C., Geiger, D. C., Villares, M. C., Schreiber, A. Z., Barone, A. A. and Branchini, M. L.: *Candida parapsilosis* fungaemia associated with implantable and semi-implantable central venous catheters and the hands of health care workers. *Diagn Microbiol Infect Dis* 1998; **30:** 243-349.
30. Costerton, J. W., Lewandowsk, Z., Caldwell, D. E., Korber, D. R. and Lappin-Scott, H. M.: Microbial biofilms. *Ann Rev Microbiol* 1995; **49:** 711-745.
31. Doughtery, S. H.: Pathobiology of infection in prosthetic devices. *Rev of Infect Dis* 1988; **10:** 1102-1117.
32. Baillie, G. S. and Douglas, L. J.: Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents. *J Antimicrob Chemother* 2000; **46:** 397-403.
33. Fridkin, S. K. and Jarvis, W. R.: Epidemiology of nosocomial fungal infections. *Clin Microb Rev* 1996; **9:** 499-511.
34. Gottlich, E., van der Lubbe, W., Lange, B., Fiedler, S., Melchert, I., Reifenrath, M., Flemming, H. C. and de Hoog, S.: Fungal flora in groundwater-derived public drinking water. *Int J Hyg Environ Health* 2002; **205:** 269-279.
35. Crnich, C. J., Safdar, N. and Maki, D. G.: The role of the intensive care unit environment in the pathogenesis and prevention of ventillator-associated pneumonia. *Resp Care* 2005; **50:** 813-836.
36. Dynesen, J. and Nielsen, J.: Branching is coordinated with mitosis in growing hyphae of *Aspergillus nidulans*. *Fungal Genet Biol* 2003; **40:** 15-24.
37. Ribes, J. A., Vanover-Sams C. L. and Baker, D. J.: Zygomycetes in human disease. *Clin Microbiol Rev* 2000; **13:** 236-301.
38. Unlu, M., Ergin, C., Cirit, M., Sahin, U. and Akkaya, A.: Molds in the homes of asthmatic patients in Isparta, Turkey. *Asian Pac J Allergy Immunol* 2003; **21:** 21-24.
39. Latgé, J.-P.: *Aspergillus fumigatus* and aspergillosis. *Clin Microbiol Rev* 1999; **12:** 210-350.

40. Hospenthal, D. R., Kwon-Chung, K. j. and Bennett, J. E.: Concentration of airborne *Aspergillus* compared to the incidence of invasive aspergillosis: lack of correlation. *Med Mycol* 1998; **36:** 165-168.
41. Gad, Z. M., Youssef, N., Sherif, A. A., Hasab, A. A., Mahfouz, A. A. and Hassan, M. N.: An epidemiologic study of the fungal skin flora among the elderly in Alexandria. *Epidemiol Infect* 1987; **99:** 213-219.
42. Oyeka, C. A. and Ugwu, L. O.: Fungal flora of human toe webs. *Mycoses* 2002; **45:** 488-491.
43. Ando, N. and Takatori, K.: Fungal flora of the conjunctival sac. *Am J Ophthalmol* 1982; **94:** 67-74.
44. Iskeleli, G., Bahar, H., Unal, M., Artunay, O., Akova, N. and Torun, M. M.: Microbiologic evaluation of frequent-replacement soft contact lenses. *CLAO* 2002; **28:** 192-195.
45. Teixeira, A. B. A., Silva, M., Lyra, L., Luz, E. A., Uno, J., Takada, H., Miyaji, M., Nishimura, K. and Schreiber, A. Z.: Antifungal susceptibility and pathogenic potential of environmental isolated filamentous fungi compared with colonizing agents in immunocompromised patients. *Mycopathologia* 2005; **160:** 129-135.
46. Khatib, R., Riederer, K. M., Ramanathan, J. and Baran, J., Jr.: Faecal fungal flora in healthy volunteers and inpatiens. *Mycoses* 2001; **44:** 151-156.
47. Cimon, B., Carrère, J., Vinatier, J. F., Chazalette, J. P., Chabasse, D. and Bouchara, J. P.: Clinical significance of *Scedosporium apiospermum* in patients with cystic fibrosis. *Eur J Clin Microbiol Infect Dis* 2000; **19:** 53-56.
48. Brenier-Pinchart, M. P., Lebeau, B., Devouassoux, G., Mondon, P., Pison, C., Ambroise-Thomas, P. and Grillot, R.: *Aspergillus* and lung transplant recipients: a mycologic and molecular epidemiologic study. *J Heart Lung Transplant* 1998; **17:** 972-979.
49. Patterson, J. E., Peters, J., Calhoon, J. H., Levine, S., Anzueto, A., Al-Abdely, H., Sanchez, R., Patterson, T. F., Rech, M., Jorgensen, J. H., Rinaldi, M. G., Sako, E., Johnson, S., Speeg, V., Halff, G. A. and Trinkle, J. K.: Investigation and control of aspergillosis and other filamentous fungal infections in solid organ transplant recipients. *Transpl Infect Dis* 2000; **2:** 22-28.
50. Leenders, A. C. A. P., Belkum, A. V., Behrendt, M., Luijendijk, A. and Verbrugh, H. A.: Density and molecular epidemiology of *Aspergillus* in air and relationship to outbreaks of *Aspergillus* infection. *J Clin Microbiol* 1999; **37:** 2343-2345.

51. Patterson, T. F.: *Aspergillus* species. *In:* Principles and practice in infectious diseases. Edited by Mandell, G. L., Bennett, J. E. and Dolin, R. Sixth edition, Elsevier Inc., Philadelphia, Pennsylvania, U. S. A., 2005; 2958-2973.
52. Deepe, G. S. Jr: *Histoplasma capsulatum*. *In:* Principles and practice in infectious diseases. Edited by Mandell, G. L., Bennett, J. E. and Dolin, R. Sixth edition, Elsevier Inc., Philadelphia, Pennsylvania, U. S. A., 2005; 3012-3026.
53. Hay, R. J.: Dermatophytosis and other superficial mycoses. *In:* Principles and practice in infectious diseases. Edited by Mandell, G. L., Bennett, J. E. and Dolin, R. Sixth edition, Elsevier Inc., Philadelphia, Pennsylvania, U. S. A., 2005; 3051-3062.
54. Cohen, J., Denning, D. W., Viviani, M. A. and EORTC Invasive Fungal Infections Cooperative Group: Epidemiology of invasive aspergillosis in European cancer centres. *Eur J Clin Microbiol infect Dis* 1993; **12:** 392-393.
55. Patel, R. and Paya, C. V.: Infections in solid-organ transplant recipients. *Clin Microbiol Rev* 1997; **10:** 86-124.
56. Espinel-Ingroff, A.: Comparison of the E-test with the NCCLS M38-P method for antifungal susceptibility testing of common and emerging pathogenic filamentous fungi. *J Clin Microbiol* 2001; **39:** 1360-1367.
57. Denning, D. W., Wenkateswarlu, K., Oakley, K. L., Anderson, M. J., Manning, N. J., Stevens, D. A., Warnock, D. W. and Kelly, S. L.: Itraconazole resistance in *Aspergillus fumigatus*. *Antimicrob Agents Chemother* 1997; **41:** 1364-1368.
58. Baddley, J. W., Stroud, T. P., Salzman, D. and Pappas, P. G.: Invasive mould infections in allogeneic bone marrow transplant recipients. *Clin Infect Dis* 2001; **32:** 1319-1324.
59. Anaissie, E., Kantarjian, H., Ro, H., Hopfer, R., Rolston, R. K., Fainstein, V. and Bodey, G.: The emerging role of *Fusarium* infections in patients with cancer. *Medicine* 1988, **67:** 77-83.
60. Berenguer, J., Rodriguez-Tudela, J. L., Richard, C., Alvarez, M., Sanz, M. A., Guztelurrutia, L. and the *Scedosporium prolificans* Spanish Study Group: Deep infections caused by *Scedosporium prolificans*. A report on 16 cases in Spain and a review of the literature. *Medicine* 1997, **76:** 256-265.
61. Girmenia, C., Luzi, G., Monaco, M. and Martino, P.: Use of voriconazole in treatment of *Scedosporium apiospermum* infection: case report. *J Clin Microbiol* 1998; **36:** 1436-1438.
62. Iwen, P. C., Rupp, M. E., Langnas, L. N., Reed, E. C. and Hinrichs, S. H.: Invasive pulmonary aspergillosis due to *Aspergillus terreus*: 12-year experience and review of literature. *Clin Infect Dis* 1998; **26:** 1092-1097.

63. Richter, S., Cormican, M. G., Pfaller, M. A., Lee, C. K., Gingrich, R., Rinaldi, M. G. and Sutton, D. A.: Fatal disseminated *Trichoderma longibrachiatum* infection in an adult bone marrow transplant patient: species identification and review of the literature. *J Clin Microbiol* 1999; **37:** 1154-1160.

64. Singh, N., Chang, F. Y., Gayowski, T. and Marino, I. R.: Infections due to dematiaceous fungi in organ transplant recipients: case report and review. *Clin Infect Dis* 1996; **24:** 369-374.

65. Yeghen, T., Fenelon, L., Campbell, C. K., warnock, D. W., Hoffbrand, A. V., Prentice, H. G. and Kibbler, C. C.: *Chaetomium* pneumonia in a patient with acute myeloid leukemia. *J Clin Pathol* 1996; **49:** 184-186.

66. Campos-Herrero, M. I., Bordes, A., Perera, A., Ruiz, M. C. and Fernandez, A.: *Trichoderma koningii* peritonitis in a patient undergoing peritoneal dialysis. *Clin Microbiol Newsletter* 1996; **18:**150-152.

67. Munoz, F. M., Demmler, G. J., Travis, W. R., Ogden, A. K., Rossmann, S. N. and Rinaldi, M. G.: *Trichoderma longibrachiatum* infection in a pediatric patient with aplastic anemia. *J Clin Microbiol* 1997; **35:** 499-503.

68. Furukawa, H., Kusne, S., D. A., Manez, R., Carrau, R., Nichols, L., Abu-Elmagd, K., Skedros, D., Todo, S. and Rinadi, M. G.: Acute invasive sinusitis due to *Trichoderma longibrachiatum* in a liver and small bowek transplant recipient. *Clin Infect Dis* 1998; **26:** 487-489.

69. Dixon, D. M. and Walsh, T. J.: Antifungal agents. *In:* Medical microbiology. Edited by Baron S. Third edition. Churchill Livingstone Inc., 1991; 959-964.

70. Meis, J. F. G. M. and Verweij, P. E.: Current management of fungal infections. *Drugs* 2001; **61 Suppl. 1:** 13-25.

71. Utz, J. P. and Drouhet, E.: Treatment of *Candida* infections. *In:* Candidasis. Edited by Bodey, G. P. and Fainstein, V. Raven Press Books, Ltd., New York, U. S. A., 1985; 253-269.

72. Vincent, J.-L., Anaissie, E., Bruining, H., Demajo, W., El-Ebiary, M., Haber, J., Hiramatsu, Y., Nitenberg, G., Nyström, P.-O., Pittet, D., Rogers, T., Sndven, P., Sganga, G., Schaller, M.-D. and Solomkin, J.: Epidemiology, diagnosis and treatment of systemic *Candida* infection in surgical patients under intensive care. *Intensive Care Med* 1998; **24:** 206-216.

73. Lopes-Berestein, G., Fainstein, V., Hopfer, R. L., Mehta, K., Sullivan, M. P., Keating, M. and Juliano, R. L.: Liposomal amphotericin B for the treatment of systemic fungal infections in patients with cancer. *J Infect Dis* 1985; **151:** 704-710.

74. Szisztémás terápia. *In:* Simon, Gy. and Török, I.: Gombás betegségek laboratóriumi diagnosztikája és terápiája. Kornétás, Budapest, Hungary, 1998; 97-111.
75. Pfaller, M. A., Diekema, D. J. and Rinaldi, M. G.: Antifungal drugs: Mechanisms of action, drug resistance, susceptibility testing and assays of activity in biological fluids. *In:* Antibiotics in laboratory medicine. Edited by Lorian, V. Fifth edition. Philadelphia, U. S. A., 2005; 226-265.
76. Cuenca-Estrella, M., Mellado, E., Díaz-Guerra, T. M., Monzón, A. and Rodríguez-Tudela, J. L.: Susceptibility of fluconazole-resistant clinical isolates of *Candida* spp. to echinocandin LY303366, itraconazole and amphotericin B. *J Antimicrob Chemother* 2000; **46:** 475-477.
77. National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A2. Second edition. Wayne, PA, NCCLS. 2002.
78. National Committee for Clinical Laboratory Standards. Method for antifungal disk diffusion susceptibility testing of yeasts: approved guideline M44-A. National Committee for Clinical Laboratory Standards, Wayne, PA., 2004.
79. National Committee for Clinical Laboratory Standards. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. Approved standard M38-A. Wayne, PA, NCCLS. 2002.
80. Rex, J. H., Pfaller, M. A., Walsh, T. J., Chaturvedi, V., Espingel-Ingroff, A., Ghannoum, M. A., Gosey, L. L., Odds, F. C., Rinaldi, M. G., Sheehan, D. J. and Warnock, D. W.: Antifungal susceptibility testing: practical aspects and current challenges. *Clin Microbiol Rev* 2001; **14:** 643-658.
81. Maxwell, M. J., Messer, S. A., Hollis, R. J., Boyken, L., Tendolkar, S., Diekema, D. J., Pfaller, M. A. and the International Fungal Surveillance Participant Group: Evaluation of Etest method for determining fluconazole and voriconazole MICs for 279 clinical isolates of *Candida* species infrequently isolated from blood. *J Clin Microbiol* 2003; **3:** 1087-1090.
82. General techniques. *In:* de Hoog, G. S., Guarro, J., Gené. J. and Figueras, M. J.: Atlas of clinical fungi. Second edition, Utrecht, Netherlands, 2000; 39-53.
83. Odds, F. C. and Bernaerts, R.: CHROMagar Candida, a new differential isolation medium for presumptive identification of clinically important *Candida* species. *J Clin Microbiol* 1994; **32:** 1923-1929.

84. Laboratory procedures. *In:* Larone, D. H.: Medically important fungi. A guide to identification. Second edition. Elsevier Science Publishing Co., Inc. Amsterdam, The Netherlands, 1987; 173-185.
85. McGinnis, M. R. and Schell, W. A.: India ink preparation procedure. *In:* Clinical microbiology procedures handbook. Edited by Isenberg, H. D. Volume 1. American Society for Microbiology, Washington, U. S. A., 1992; 6.5.
86. McGinnis, M. R. and Pasarell, L.: Yeast identification using morphology. *In:* Clinical microbiology procedures handbook. Edited by Isenberg, H. D. Volume 1. American Society for Microbiology, Washington, U. S. A., 1992; 6.9.
87. McGinnis, M. R. and Pasarell, L.: Mould identification. *In:* Clinical microbiology procedures handbook. Edited by Isenberg, H. D. Volume 1. American Society for Microbiology, Washington, U. S. A., 1992; 6.11.
88. Methods. *In:* St-Germain, G. and Summerbell, R.: Identifying filamentous fungi. A clinical laboratory handbook. Star Publishing Company, Belmont, California, U. S. A., 1996; 243-269.
89. Hazen, K. C., Baron, E. J., Colombo, A. L., Girmenia, C., Sanchez-Sousa, A., del Palacio, A., de Bedout, C., Gibbs, D. L. and The Global Antifungal Surveillance Group: Comparison of the susceptibilities of *Candida* spp. to fluconazole and voriconazole in a 4-year global evaluation using disk diffusion. *J Clin Microbiol* 2003; **41,** 5623-5632.
90. Pfaller, M. A., Diekema, D. J., Messer, S. A., Boyken, L. and Hollis, R. J.: Activities of fluconazole and voriconazole against 1,586 recent clinical isolates of *Candida* species determined by broth microdilution, disk diffusion and Etest methods: report from the ARTEMIS Global Antifungal Susceptibility Program, 2001. *J Clin Microbiol* 2003; **41:** 1440-1446.
91. Etest technical guide No. 4: Antifungal susceptibility testing of yeasts. Solna, Sweden, AB biodisk.
92. Etest technical guide No. 10: Antifungal susceptibility testing of moulds. Solna, Sweden, AB biodisk.
93. McGinnis, M. R. and Rinaldi, M. G.: Antifungal drugs: Mechanisms of action, drug resistance, susceptibility testing and assays of activity in biological fluids. *In:* Antibiotics in laboratory medicine. Edited by Victor, L. Second edition. Baltimore, U. S. A., 1986; 223-281.
94. Miller, S. A., Dykes, D. D. and Polesky, H. F.: A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res* 1988; **16:** 1215-1218.

95. Laboratory diagnosis. *In:* Kwon, K. J. and Bennett, J. E.: Medical mycology. Lea and Febiger, Philadelphia, Pennsylvania, U. S. A., 1992; 44-71.

96. Ahmad, S., Khan, Z., Mokaddas, E. and Khan, Z. U.: Isolation and molecular identification of *Candida dubliniensis* from non-human immunodeficiency virus-infected patients in Kuwait. *J Med Microbiol* 2004; **53:** 633-637.

97. Tintelnot, K., Haase, G., Sheibold, M., Bergmann, F., Staemmler, M., Franz, T. and Naumann, D.: Evaluation of phenotypic markers for selection and identification of *Candida dubliniensis*. *J Clin Microbiol* 2000; **38:** 1599-1608.

98. Khan, Z. U., Ahmad, S., Mokaddas and E., Chandy, R.: Tobacco agar, a new medium for differentiating *Candida dubliniensis* from *Candida albicans*. *J Clin Microbiol* 2004; **42:** 4796-4798.

99. Al-Sweih, N., Ahmad, S., Khan, Z. U., Khan, S. and Chandy, R.: Prevalence of *Candida dubliniensis* among germ tube-positive *Candida* isolates in a maternity hospital in Kuwait. *Mycoses* 2005; **48:** 347-351.

100. Acikgoz, Z. C., Sancak, B., Gamberzade, S. and Misirlioglu, M.: Prevalence of *Candida dubliniensis* among the stored vaginal *Candida* isolates in a Turkish hospital. *Mycoses* 2004; **47:** 393-396.

101. De Vos, M. M., Cuenca-Estrella, M., Boekhout, T., Theelen, B., Matthijs, N., Bauters, T., Nailis, H., Dhont, M. A., Rodriguez-Tudela, J. L. and Nelis, H. J.: Vulvovaginal candidiasis in a Flemish patient population. *Clin Microbiol Infect* 2005; **11:** 1005-1011.

102. Yapar, N., Uysal, U., Yucesoy, M., Cakir, N. and Yuce, A.: Nosocomial bloodstream infections associated with *Candida* species in a Turkish university hospital. *Mycoses* 2006; **49:** 134-138.

103. Bedini, A., Venturelli C., Mussini, C., Guaraldi, G., Codeluppi, M., Borghi, V., Rumpianesi, F., Barchiesi, F. and Esposito, R.: Epidemiology of candidaemia and antifungal susceptibility patterns in an Italian tertiary-care hospital. *Clin Microbiol Infect* 2006; **12:** 75-80.

104. Almirante, B., Rodríguez, D., Park, B. J., Cuenca-Estrella, M., Planes, A. M., Almela, M., Mensa, J., Sanchez, F., Ayats, J., Gimenez, M., Saballs, P., Fridkin, S. K., Morgan, J., Rodriguez-Tudela, J. L., Warnock, D. W., Pahissa, A. and the Barcelona Candidemia Project Study Group: Epidemiology and predictors of mortality in cases of *Candida* bloodstream infection: Results from population-based surveillance, Barcelona, Spain, from 2002 to 2003. *J Clin Microbiol* 2005; **43:** 1829-1835.

105. Samra, Z., Yardeni, M., Peled, N. and Bishara, J.: Species distribution and antifungal susceptibility of *Candida* bloodstream isolates in a tertiary medical center in Israel. *Eur J Clin Microbiol Infect Dis* 2005; **24:** 592-595.

106. Aquino, V. R., Lunardi, L. W., Goldani, L. Z. and Barth, A. L.: Prevalence, susceptibility profile for fluconazole and risk factors for candidemia in a tertiary care hospital in Southern Brazil. *Braz J Infect Dis* 2005; **9:** 411-418.

107. Arendrup, M. C., Fuursted, K., Gahrn-Hansen, B., Jensen, I. M., Knudsen, J. D., Lundgren, Schønheyder, H. C. and Tvede, M.: Seminational surveillance of fungemia in Denmark: Notably high rates of fungemia and numbers of isolates with reduced azole susceptibility. *J Clin Microbiol* 2005; **43:** 4434-4440.

108. Martin, D., Persat, F., Piens, M. A. and Picot, S.: *Candida* species distribution in bloodstream culture in Lyon, France, 1998-2001. *Eur J Clin Microbiol Infect Dis* 2005; **24:** 329-333.

109. Pfaller, M. A., Diekema, D. J., Jones, R. N., Sader, H. S., Fluit, A. C., Hollis, R. J., Messer, S. A. and the SENTRY Participant Group: International surveillance of bloodstream infections due to *Candida* species: frequency of occurrence and in vitro susceptibilities to fluconazole, ravuconazole and voricaonzole of isolates collected from 1997 through 1999 in the SENTRY antimicrobial surveillance program. *J Clin Microbiol* 2001; **39:** 3254-3259.

110. Pfaller, M. A., Jones, R. N., Doern, G. V., Sader, H. S., Hollis, R. J., Messer and The SENTRY Participant Group: International surveillance of bloodstream infections due to *Candida* species: Frequency of occurrence and antifungal susceptibilities of isolates collected in 1997 in the United States, Canada and South America for the SENTRY Program. *J Clin Microbiol* 1998; **36:** 1886-1889.

111. McNeil, M. M., Nash, S. L., Hajjeh, R. A., Phelan, M. A., Conn, L. A., Plikaytis, B. D. and Warnock, D. W.: Trends in mortality due to invasive mycotic diseases in the United States, 1980-1997. *Clin Infect Dis* 2001; **33:** 641-647.

112. White, P. L., Shetty, A. and Barnes, R. A.: Detection of seven *Candida* species using the Light-Cycler system. *J Med Microbiol* 2003; **52:** 229-238.

113. Tirodker, U. H., Nataro, J. P., Smith, S., LasCasas, L. and Fairchild, K. D.: Detection of fungemia by polymerase chain reaction in critically ill neonates and children. *J Perinat* 2003; **23:** 117-122.

114. Xie, L., Dong, X. and Shi, W.: Treatment of fungal keratitis by penetrating keratoplasty. *Br J Ophthalmol* 2001; **85:** 1070-1074.

115. Gopinathan, U., Garg, P., Fernandes, M., Sharma, S., Athmanathan, S. and Rao, G. N.: The epidemiological features and laboratory results of fungal keratitis: a 10-year review at a referral eye care center in South India. *Cornea* 2002; **21:** 555-559.

116. Wong, T. Y., Fong, K. S. and Tan, D. T.: Clinical and microbial spectrum of fungal keratitis in Singapore: a 5-year retrospective study. *Int Ophthalmol* 1997; **21:** 127-130.

117. Rondeau, N., Bourcier, T., Chaumeil, C., Borderie, V., Touzeau, O., Scat, Y., Thomas, F., Baudouin, C., Nordmann, J. P. and Laroche, L.: Fungal keratitis at the Centre Hospitalier National d'Ophtalmologie des Quinze-Vingts: retrospective study of 19 cases [in French]. *J Fr Ophtalmol* 2002; **25:** 890-896.

118. Thomas, P. A., Abraham, D. J., Kalavathy, C. M. and Rajasekaran, J.: Oral itraconazole therapy for mycotic keratitis. *Mycoses* 1988; **31:** 271-279.

119. Dursun, D., Fernandez, V., Miller, D. and Alfonso, E. C.: Advanced *Fusarium* keratitis progressing to endophthalmitis. *Cornea* 2003; **22:** 300-303.

120. Widden, P.: The effects of temperature on competition for spruce needles among sympatric species of *Trichoderma*. *Mycologia* 1984; **76:** 873-883.

121. Kredics, L., Antal, Zs., Szekeres, A., Manczinger, L., Dóczi, I., Kevei, F. and Nagy, E.: Production of extracellular proteases by human pathogenic *Trichoderma longibrachiatum* strains. *Acta Microbiol Immunol Hung* 2004; **51:** 283-295.

122. Pfaller, M. A., Messer, S. A., Mills, K. and Bolmström, A.: *In vitro* susceptibility testing of filamentous fungi: Comparison Etest and reference microdilution methods for determining itraconazole MICs. *J Clin Microbiol* 2000; **38:** 3359-3361.