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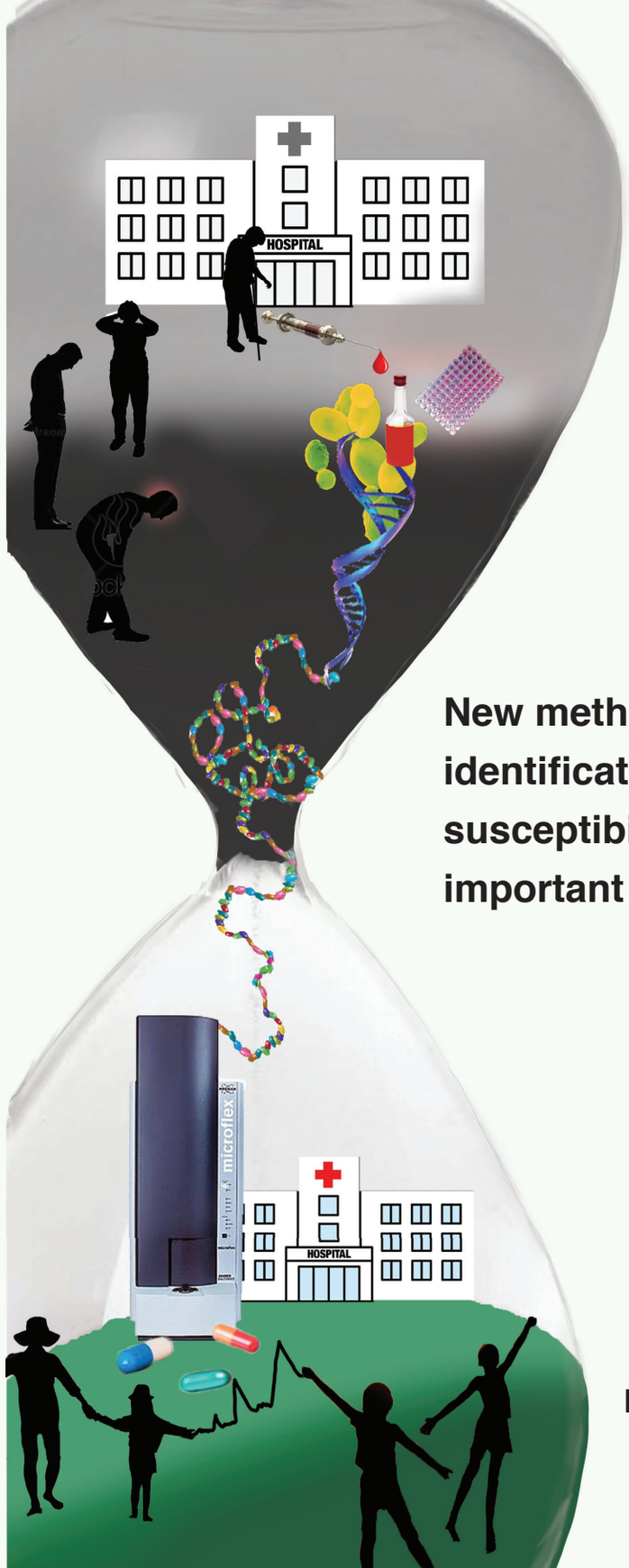

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Schematic representation of the influence of time, diagnostic and antifungal susceptibility testing methods on human´s life

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Chapter 1

Introduction

Mycology refers to the part of biology that studies fungi in terms of genetics, biochemistry, biodiversity, taxonomy, their biotechnological applications to make medicines and food for humans, as well as their role in threatening plants, animals and human life (1–4). Fungi are a large group of heterotrophic eukaryotes with a chitinous cell wall. They have a vast metabolically variety, inhabit different ecological niches, and interact with many other living organisms (5). It is estimated that nearly 4 million fungal species exist that belong to seven phyla: Microsporidia, Chytridiomycota, Blastocladiomycota, Neocallimastigomycota, Glomeromycota, Ascomycota, and Basidiomycota (6, 7). Those species of yeasts and filamentous fungi that are a threat for human health mostly belong to the Ascomycota and Basidiomycota phyla (1, 6, 8). Fungi are important for a wide range of scientific and industrial processes and play an important role in natural and man-made ecosystems. They can grow as saprotrophs, symbionts, commensals, and parasites or pathogens (9, 10).

Ascomycota is a division of subkingdom Dikarya and is the largest phylum of fungi with more than 64,000 species, including yeasts and molds (6). The species that belong to this phylum reproduce sexually by the formation of ascospores, and have a significant influence on human life in different ways. For instance, some species are used in the industry, e.g. for baking, food fermentations, to produce antibiotics, and as heterologous producers of drugs, such as insulin. Several species are also important causing diseases to human, animal, and plants, to spoil food, or to make poisonous substances. Ascomycota have three subphyla, namely Pezizomycotina, Saccharomycotina, and Taphrinomycotina. The subphylum Saccharomycotina includes yeasts that are considered as one of the most important microorganisms in the clinic. Yeasts are morphologically different and have several nutritional habitats that allow them to survive in different environments. They have also different living conditions and life cycles. Yeasts play

a key role in human life either positively in food and beverage production, and fermentation and they are used to produce medicines, but they can also act negatively by causing various infections (1, 6, 11).

Basidiomycota is the other division of subkingdom Dikarya that contains about 30,000 species and the majority of them are filamentous fungi. These filamentous fungi are composed of hyphae and reproduce sexually by basidiospores. This group of fungi is known for their different biological functions in nature and agricultural as symbionts, decomposers, and pathogens. The important human pathogenic yeast, *Cryptococcus neoformans*, also belongs to this group (6, 9, 10). Besides the *Cryptococcus neoformans* and *Cryptococcus gattii* species complex that are clinically important, the majority of the Basidiomycota species are involved in carbon recycling and ecosystem functioning, e.g. by the decomposition of plant biomass and as mycorrhizal symbionts. These species produce a wide range of products from deadly toxins and hallucinogens, but also natural products and enzymes that are very useful for society (9, 10).

Fungi can synchronously live in a microbe-host relationship without any pathogenic characteristic. *Candida albicans*, for instance, occurs as commensal in the intestines of healthy humans (12), but this species is also responsible for many infections. Different factors play a role in the transitions of fungi into a pathogenic state. For plant pathogens, these are changes of the climate, competition and predation, and human`s manipulations of nature (13). For human pathogens, or human opportunistic pathogens to be more correct, the immune status of the host is a key factor (12).

More than a billion people are infected per year by different fungal diseases worldwide (4, 7, 14). Among them, more than 150 million people are suffering from serious infectious diseases, and more than 1.6 million people die per year due to fungal infections (4, 7, 14). The epidemiology of fungal infections is associated with the type of patients and the ecology of the

respective opportunistic fungus (7). If fungal infections occur concomitantly with diseases suppressing the immune system they can be a threat to a human´s life. Diseases, like HIV/AIDS, cancer, organ transplantation, tuberculosis, chronic obstructive pulmonary disease (COPD), asthma, and corticosteroid therapies can stimulate a moderate fungal infection into severe disease (4, 14–16). Among several fungal taxa, species of *Aspergillus*, *Candida*, *Cryptococcus*, *Histoplasma*, *Mucor*, *Pneumocystis*, and *Rhizopus* are responsible for the majority of human fungal infections (17–19). It has been estimated that more than one million deaths occur annually due to fungal infections caused by *Candida*, *Cryptococcus*, and *Aspergillus* species (7). Thus far, several studies illustrated the global estimates of the respective fungal diseases and the results are summarized in Table 1 (4). Briefly, the prevalence of chronic pulmonary aspergillosis, invasive aspergillosis, and invasive candidiasis has been approximately reported to be 3,000,000, 250,000, and 700,000 cases per year, respectively. The prevalence of cryptococcal meningitis complicating HIV/AIDS is estimated over 223,100 cases per year (4, 20–22). Among fungal infectious diseases, candidiasis has the highest number with tens of millions of mucosal infections and about 700,000 cases of invasive candidiasis per year (4, 14). Hereupon, in this dissertation, *Candida* species and candidiasis are the main focus.

Candidiasis

In the last 40 years, the number of candidiasis cases has been rising and became one of the greatest public health problems. Candidiasis is a fungal infection caused by *Candida* species (23, 24). *Candida* species are opportunistic fungi that may cause infections ranging from mild irritation or superficial infections in immunocompetent individuals to severe systematic disease in immunocompromised patients (23). Invasive candidiasis does not only occur in immunocompromised patients, but it has also been reported in hematological, transplant and

intensive care unit (ICU) patients. Invasive candidiasis is often diagnosed in cancer patients, hence it is mostly reported from wealthy countries (7, 23, 24).

Table 1. Global estimates of fungal diseases. Data from Bongomin *et al.* (2017) [4].

	Fungal Disease	Annual Incidence	Global Burden
Superficial	Skin, hair, nail		~1,000,000,000
	Fungal keratitis		~1,000,000
Mucosal	Oral candidiasis	~2,000,000	
	Oesophageal candidiasis	~1,300,000	
	Vulvovaginal candidiasis		~134,000,000
Allergic	Allergic bronchopulmonary aspergillosis in asthma		~4,800,000
	Allergic bronchopulmonary aspergillosis in cystic fibrosis		~6675
	Severe asthma with fungal sensitisation		~6,500,000
	Fungal rhinosinusitis		~12,000,000
Chronic severe	Chronic pulmonary aspergillosis		~3,000,000
	Mycetoma		~9000
	Chromoblastomycosis		>10,000
	Coccidioidomycosis		~25,000
	Paracoccidioidmycosis		~4000
	Blastomycosis		~3000
	Histoplasma infection	~500000	~25,000
	Sporotrichosis	>40,000	
Acute invasive	Invasive candidiasis	~750,000	
	Invasive aspergillosis	>300,000	
	Cryptococcosis in AIDS	~223,000	
	Mucormycosis	>10,000	
	Disseminated histoplasmosis	~100,000	
	Talaromycosis	~8000	

In 2009, Vincent and coauthors described the most prevalent clinical pathogens involved in developing sepsis, including *Candida* yeast that cause candidiasis. Based on their report, invasive candidiasis is estimated to be the third most common infection worldwide, and the second in the USA. They also stated that patients had to stay much longer in the ICU resulting in increased costs of hospitalization due to candidemia (25). Nosocomial bloodstream

infections (BSI) can be caused by bacteria, fungi, viruses, and parasites. BSIs largely contribute to an increase of mortality rates. Several studies showed that bacterial infections caused by Gram-positive (e.g. *Staphylococcus aureus*) and Gram-negative bacteria (e.g. *Escherichia coli*) are the main causes of BSI, followed by invasive candidiasis (26–28).

Thus far, it has been estimated that 79% of invasive fungal infections are related to *Candida* spp., with the highest incidence occurring amongst transplant patients (29, 30). The mortality rate of infections caused by *Candida* spp. has been reported as approximately 39% worldwide, while in the USA it is up to 50% (4, 23, 31, 32). So far, more than 1,500 Saccharomycotina yeasts have been described (33), and, fortunately, only some of them are significant in the clinic. *Candida albicans* is the most common *Candida* species causing of 90–100% of mucosal infections and 40-70% invasive ones (23, 34, 35).

Although *C. albicans* (46%) remains the most frequently encountered *Candida* species, the increasing incidence of non-albicans *Candida* (NAC) species has changed the epidemiology and outcomes of invasive candidiasis (35) that worrying the field. The most prevalent NAC-species are *C. glabrata*, followed by *C. parapsilosis*, *C. tropicalis*, *Pichia kudriavzevii* (= *C. krusei*), *Meyerozyma guilliermondii* (= *C. guilliermondii*), *Clavispora lusitaniae* (= *C. lusitaniae*), *Kluyveromyces marxianus* (= *C. kefyr*), *Yarrowia lipolytica* (= *C. lipolytica*), *Debaryomyces hansenii* (= *C. famata*), *Pichia inconspicua* (*C. inconspicua*), *Diutina rugosa* (= *C. rugosa*), *C. dubliniensis*, and *Pichia norvegensis* (= *C. norvegensis*) (24, 35, 36). Prior studies have reported that the proportion of NAC-species has risen from 10-40% up to 35-65% in the last decades (35).

The geographical distribution of candidiasis caused by NAC-species varies. For instance, *C. glabrata* is highly prominent in Australia, Canada, the USA, and Central and Northern Europe. *C. parapsilosis* is a predominant species in Africa, China, Japan, and South America (37, 38), and *C. tropicalis* is commonly distributed in tropical regions, like the Pacific-Asia region,

Brazil, and recently also in Europe (39). The number of studies on NAC-species is growing and this highlights their increasing role in developing infections and resistance to different antifungals (40). Invasive candidiasis caused by NAC-species usually occurs among intensive care or in immunocompromised patients, and HIV-positive individuals (35, 40). However, the incidence of NAC in cancer patients, especially those with hematological malignancies and bone marrow transplant (BMT) recipients, is higher than patients that stay in the ICU or surgical wards, as well as children and HIV-positive patients (40, 41). Among the NAC-species, *C. glabrata* (33.3%) and *C. tropicalis* (20.3%) are the two most commonly reported species in patients with BSI (42–44). However, in some countries, like Portugal and Spain, invasive candidiasis caused by NAC-species is frequently due to *C. parapsilosis* followed by *C. glabrata* (38). The mortality rate of NAC-species of 15-35% is similar to that of *C. albicans*, and associated with the patient´s health condition and the NAC-species involved. For instance, infections by *C. glabrata* and *C. tropicalis* have a high mortality rate (40-70%), while *C. parapsilosis* is known to be a less harmful species with a mortality rate of 8% (40, 45).

Candida parapsilosis is a complex of three different species: *Candida parapsilosis sensu stricto*, *Candida metapsilosis*, and *Candida orthopsilosis*. Infections by *C. parapsilosis* usually originate from an exogenous source that are more common among children than older people (46). The infections caused by this species are associated with cancer patients, neonates and young adults in ICUs (46). A particular feature of *C. parapsilosis* that is relevant to its ability to cause disease, is that this species forms extensive biofilms, also on medically implanted devices. Adhesion is the first step of this yeast to develop biofilms, and this occurs via different cell wall proteins (CWPs), such as Als-, Hwp-, or Hyr-like proteins to adhere to host cells, and Pgk (phosphoglycerate kinase) and Eno1 (enolase 1) for the adhesion of *C. parapsilosis* cells to silicone-made materials (47). *C. tropicalis* is another common NAC-species that is common in patients suffering from hematological malignancies, septic shock, and emboli (48). Like

other *Candida* species, the production of proteinase and phospholipase, and biofilm-formation are known as the main virulence factors for this yeast species. Secreted aspartyl proteinase 5 and 9 (*SAP5* and *SAP9*) are two virulence factors that play a key role in the pathogenicity of *C. tropicalis* and are also presented on the surface of the yeast cell wall before they are invaded by macrophages after phagocytosis of yeast cells. *Pichia kudriavzevii* (= *C. krusei*) remains as one of the *Candida* spp. with the lowest prevalence (2%) among yeast-related infections, but it has a higher 90-day mortality rate (53.6%) than more common *Candida* species, such as *C. albicans* (49).

Another concern related to NAC-species is their reduced sensitivity to antifungals. Although the majority of NAC-species are still susceptible to most of the commonly used antifungals, some NAC-species showed lower antifungal susceptibility (40, 50). Whether *in vitro* resistance of NAC-species to a special antifungal is acquired after exposure or whether the species are inherently resistant, is also important. Results from different studies indicate that the incidence rate of azole resistance is increasing among NAC-species (51, 52). In 2009, Leroy and collaborators reported that two-third of invasive candidiasis caused by NAC-species detected in the ICU were resistant to fluconazole (53). Some species, like *C. glabrata* and *Pichia kudriavzevii*, are inherently less susceptible to fluconazole, and resistance to amphotericin B is acquired by these species after exposure to this antifungal, and this raises epidemiological and therapeutic concerns (40, 54). The majority of *C. parapsilosis* isolates are susceptible to different antifungals, but it has been reported that some clinical isolates have a lower susceptibility to azoles and echinocandins (38). Azole resistant *C. tropicalis* isolates have been reported worldwide, but the minimal inhibitory concentrations (MIC) values remain below the threshold considered as a threat for human life (51, 55). There is a controversy in the number of reported NAC-species resistant to different antifungals between data acquired from different geographical distributions. For instance, in a recent study performed in Thailand, the

susceptibility of 85 NAC species isolates comprising *C. glabrata*, *C. tropicalis*, *C. kefyr*, and *C. parapsilosis* to azoles were tested, and only two *C. glabrata* isolates were found to be resistant to all three azoles tested, whereas all clinical isolates of *C. tropicalis* and *C. kefyr* were susceptible to all three azoles. Investigators found many silent mutations in *CgERG11*, *CgERG3*, and *CgPDR1* genes in different species, particularly *C. glabrata* (52). In another study, the susceptibility of 2936 isolates from different species collected worldwide was tested for susceptibility to azoles and echinocandins. Few isolates were detected to be resistant to echinocandins with the highest number occurring in *C. glabrata*. Resistant isolates to azoles occurred in several species, like *C. dubliniensis*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *Cl. lusitaniae, M. guilliermondii*, and *P. kudriavzevii*, (51). Recently, *C. glabrata* isolates resistant to echinocandins have been increasingly reported and this is becoming a clinical concern (56, 57). Nunnally and collaborators (2019) tested 89 *C. glabrata* isolates against echinocandins. Surprisingly, their results showed that all isolates were resistant to at least one of the echinocandins (58). Another publication by Rivero-Menendez and collaborators (2019) confirmed the increasing number of *C. glabrata* isolates resistant to echinocandins. They also found that the majority of *C. glabrata* isolates became resistant after exposure to the drugs (59).

In addition to the above *Candida* spp., newly emerging NAC-species are another important subject in the clinic. *Candida auris* is such an example. This species was found firstly in Japan in 2009, and within 10 years it has been dispersed at a global scale. This species adapts to the different environmental conditions and this may have contributed to its rapid spread (60, 61). *C. auris* causes invasive infections and has a high mortality rate of c. 35.0% among high-risk patients, such as immunocompromised individuals and those with broad-spectrum use of antimicrobial drugs, as well as patients with invasive devices, including tracheostomy tubes or percutaneous endoscopic gastrostomy tubes. One of the main features of this yeast species is that most of the isolates are resistant to azoles and they show reduced susceptibility to

amphotericin B (61–63). The treatment of *C. auris* is turning into a major challenge due to emerging isolates that are resistant to echinocandins (64, 65). *Pichia inconspicua* and *Pichia norvegensis* (= *C. norvegensis*) are two other examples of newly emerging *Candida* spp. in the clinic. These two species are phylogenetically closely related and have been recently isolated from respiratory and digestive areas from patients in different hospitals (63, 64). *Pichia inconspicua* seems more common than *Pichia norvegensis* with a significant 10-fold increase in developing an infection in the last 10 years (66). Both species are susceptible to the available antifungals, but a growing number of isolates showed reduced susceptibility to fluconazole (66, 67). In summary, due to the increasing number of diseases related to a suppressed human immune system, other new opportunistic *Candida* species are discovered that may turn into a serious threat to human life. Two main clinically important yeast pathogens, namely *C. albicans* and *C. glabrata*, and the emerging *C. auris*, were studied in this research project and are described in detail below.

Candida albicans

Candida albicans is known as the most predominant *Candida* species causing candidemia with an incidence ranging from 30 to 60%, depending on the different geographical locations (68–70). *C. albicans* is a commensal yeast occurring in the human body where it is found in the oral cavity, gastrointestinal tract, skin, and vagina (71, 72). Changes in the host status, such as immunodeficiency, antibiotic use, stress, and environmental changes of the natural niches of *Candida* species in the human body, like a reduction of pH of the vagina, may lead to an infection caused by this species (73, 74). This yeast is responsible for a wide range of clinical manifestations, ranging from mild superficial infections (e.g. skin, oral, and vaginal candidiasis) to severe invasive systematic infections. In immunocompetent individuals, the immune system keeps this microorganism from causing superficial mucosal infections.

However, in immunocompromised and patients suffering from critical diseases, like neonates, transplant and cancer patients, this microorganism turns into its invasive form and it may cause life-threatening systemic diseases (72, 75, 76). *C. albicans* is the most frequent yeast detected from BSIs with a high mortality risk of 50% (68, 73, 77, 78). A number of factors play a role in *C. albicans*-related infections, namely the host immune response, the anatomical site of infection, the virulence of the isolate, including its dimorphic behavior, and the expression of specific genes, such as hypha-specific genes (73, 74). *C. albicans* has a variable morphology that allows this yeast species to interact with the human cells (75, 79). The transition between yeast cells, pseudohyphae (i.e. adhering and branched or unbranched chains of elongated yeast cells), and hyphae (i.e. multicellular filamentous arranged series of cells that are usually branched) is associated with the host environment (72, 79, 80). Next to the morphological transition that is involved in the pathogenicity of *C. albicans*, also some other virulence factors, like iron acquisition, the production of hydrolytic enzymes, such as aspartic proteinases (Saps), phospholipases (PL), and lipases (Lip) are implicated (81). Furthermore, *C. albicans* can develop different colony morphologies, e.g. a rough colony morphology with filamentation after prolonged incubation, and a smooth colony morphology on chocolate agar (82, 83). Hyphae are the invasive form and can penetrate the mucous membranes and then invade into the tissue. Invasion occurs via two mechanisms: 1) the production of lytic enzymes, such as secreted aspartyl proteinases (SAPs), and 2) induction of epithelial cell endocytosis (84). The SAP enzymes digest the surface of the epithelial cell and make it possible for *C. albicans* cells to invade the tissue cells. In the other mechanism, epithelial cells produce pseudopods and induce endocytosis of *C. albicans* cells (84). The final step is that *C. albicans* enters the bloodstream, causing invasive candidiasis. In a potential patient, invasive candidiasis occurs as *C. albicans* enters the bloodstream via penetration of the gastrointestinal mucosa or directly via an intravascular catheter. Expression of adhesion proteins, like Als3 and Hwp1, on the

hyphal cell walls leads to more adherence of hypha to host cells, if compared to the yeast cells, and this may accelerate the process of invasion to the bloodstream (84). However, *C. albicans* tries to escape from the human immune system via the formation of hyphae in the phagosome that gives the yeast the ability to escape phagocytosis and kill the macrophage (72, 74, 85). Genomic instability plays a vital role in the pathogenicity and resistance of *C. albicans* to different antifungals (73, 79, 86). Genomic variations (e.g. mutations) in *C. albicans* occur when it is exposed to stress. Gene mutations correlate with a faster growth rate (87). Furthermore, a high rate of amino acid substitutions in different genes, depending on the used antifungals, contributes to the expression of different proteins in the cell membrane/wall of *C. albicans* resulting in an increase of cell adhesion and host interactions. These properties plus the morphological changes may increase the pathogenicity of this yeast (87).

Resistance to antifungal drugs of *C. albicans* has received considerable attention. Depending on the antifungals used, different resistance mechanisms occur: **a.** target overexpression, **b.** target alteration, **c.** drug sequestration, **d.** enhanced drug efflux, and **e.** blocking of antifungal drug entry (88). Many studies screened for mutations in specific genes of *C. albicans* as a major cause of resistance to different antifungals (Figure 1) (88–91). Among all available antifungals, fluconazole is widely prescribed against *C. albicans* because it is affordable, effective and shows predictable pharmacokinetics. The drug is well tolerated and suitable for treatment and use as a prophylaxis for most patients (92, 93). Mukherjee *et al.* (2003) tested the possible variations between planktonic cells and biofilm forms of *C. albicans* isolates which are resistant to azoles. After 24h, biofilms formed by both parent and mutant *C. albicans* isolates were equally resistant to azoles. Their analyses showed that ergosterol levels were significantly decreased in *C. albicans* biofilms compared to the planktonic cells. Furthermore, the function of the efflux pumps was the same between biofilm and planktonic cells after 24h. Expression of *CDR* and *MDR1* genes was observed in all the developmental phases of *C. albicans* biofilms,

while their expression occurred only at the 12- and 48-h time points of the planktonic cells (90).

The following factors are known to be related to resistance of azoles: **a.** point mutations in the *ERG11* gene, including three active hotspot regions, that is involved in ergosterol biosynthesis (91, 94), **b.** overexpression of the *ERG11* gene (91, 95), **c.** point mutations in the *ERG3* gene involved in the ergosterol biosynthesis enzyme (91, 96), **d.** overexpression of genes encoding the *Candida* Drug-Resistance (*CDR*) pump, a member of ATP-binding cassette (91, 97, 98), and **e.** overexpression of the gene encoding the Major Facilitator Superfamily (*MFS*) efflux pump (91, 97, 98). Furthermore, data from several studies indicated that multidrug-resistant *C. albicans* isolates harbor mutations in several genes encoding *CDR1*, *CDR2*, and *MDR1* proteins that impact the efflux pumps and transport drugs of the yeast cells (Figure 1) (90, 91).

The use of echinocandins is the best alternative treatment for *C. albicans* infections caused by isolates that are resistant to azoles. The number of *C. albicans* isolates that are susceptible to anidulafungin, micafungin and caspofungin is still higher than that are susceptible to azoles, particularly fluconazole (99). Therefore, echinocandins are considered as the empirical antifungal drugs to control invasive candidiasis (99). These drugs have been also described as one of the preferred treatments to prevent and cure catheter-associated infections in animal models (99–101). However, in recent years, *C. albicans* isolates with acquired resistance to echinocandins have been reported globally (102). Decreased affinity to the target of echinocandins, i.e. glucan synthase, by *C. albicans* is usually linked to point mutations in two hot spots of the *FKS1* gene, namely HS1 (aa 641-649), and HS2 (aa 1345-1365). In clinical practice, the most common amino acid substitutions that cause failed therapy or poor response to the treatment are S645F, S645P, S645Y, F641S, and F641L of HS1 for *FKS1* (103, 104). It is well established that not only mutations in the two hotspots of the *FKS1* gene, but also other factors are related to the decreasing susceptibility of *C. albicans* isolates to echinocandins (105,

106). For instance, *CAT1* is a single copy gene encoding cytoplasmic catalase, which is an enzyme playing a role against oxidative stress. Román *et al.* (2016) demonstrated that overexpression of *CAT1* is associated with an increased tolerance to caspofungin of *C. albicans* isolates, while it does not impact the susceptibility of fungicidal azoles or amphotericin B (105). Furthermore, a recent study has found a connection between chromosome 5 (Ch5) and the acquired tolerance to echinocandins (106). Chromosome 5 carries multiple genes, and mutations in the Ch5 genes *CHT2*, *CSU51*, or *PGA4* lead to an increased tolerance to caspofungin and anidulafungin (106). Additionally, these mutations reduce the 1,3-β-glucan content and increase the chitin contents of the cell wall. Thus, it causes remodeling of the cell wall of *C. albicans*, and accordingly, lead to an increase of the MIC of echinocandins (106). More recently, Xie *et al.* (2017) demonstrated that the transcription factor Cas5 plays a key role in the cell cycle dynamics and responses to echinocandins in *C. albicans*. They showed that Cas5 has different individual transcriptional targets that are activated under the presence of antifungals and/or stress. Under the presence of echinocandins, Cas5 changes the regulation of the genes involved in the cell wall integrity. Accordingly, by inhibiting the gene encoding β-1,3-glucan synthesis, β-1,3-glucan production in the cell wall is hindered and caused isolates to be resistant to echinocandins (107).

C. albicans cells are usually susceptible to amphotericin B (AMB). However, several studies have shown a positive correlation between biofilm formation and increased MICs to AMB (108–110). Surface-adherent extracellular matrices of the biofilm prohibit the interaction of AMB to the target and, subsequently, increase resistance of *C. albicans* isolates to this drug (110, 111). *C. albicans* breakpoints against azoles, echinocandins and amphotericin B established by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methods are shown in Table 2 (112, 113).

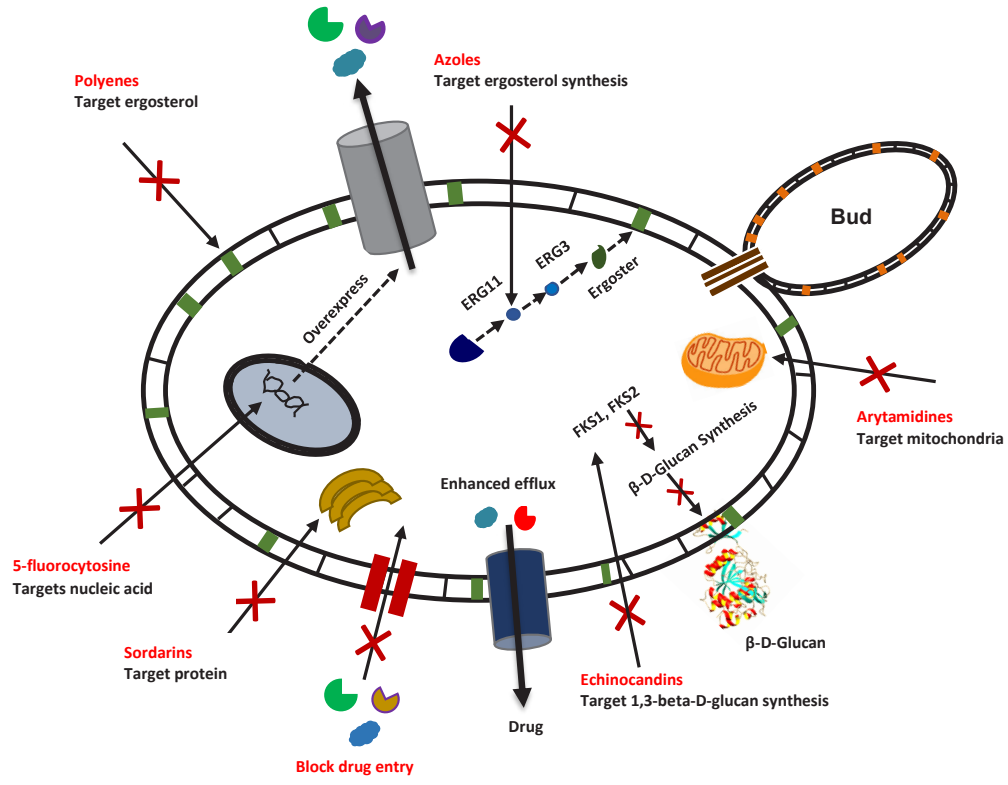


Figure 1. Mechanism of developing antifungal resistance in yeasts. There are five different classes of antifungals, and the most common ways of developing antifungals resistance are shown in this figure. This figure is based on Cui *et al.* (2015) [88].

Table 2. Suggested Minimum Inhibitory Concentration (MIC) ranges based on the CLSI and EUCAST microdilution methods against anidulafungin, micafungin, caspofungin, and fluconazole for *C. albicans* and *C. glabrata* (109, 110). Susceptible species are shown by (S) and resistant species are shown by (R).

Candida species	Methods	Anidulafungin	Micafungin	Caspofungin	Fluconazole
C. albicans	CLSI	S ≤ 0.25 I= 0.5 R≥ 1	S ≤ 0.25 I= 0.5 R≥ 1	S ≤ 0.25 I= 0.5 R≥ 1	S ≤ 2 SDD= 4 R≥ 8
	EUCAST	S ≤ 0.032 R > 0.032	S ≤ 0.016 R > 0.016	Note[1]	S ≤ 2 R > 4
C. glabrata	CLSI	S ≤ 0.12 I= 0.25 R≥ 0.5	S ≤ 0.06 I= 0.12 R≥ 0.25	S ≤ 0.12 I= 0.25 R≥ 0.5	S (-) SDD≤ 32 R≥ 64
	EUCAST	S ≤ 0.064 R > 0.064	S ≤ 0.032 R > 0.032	Note[1]	S ≤ 0.001 R > 16
C. auris	CLSI	Note[2]	Note[2]	Note[2]	Note[2]
	EUCAST	Note[2]	Note[2]	Note[2]	Note[2]

Note[1]: The breakpoints against caspofungin for different *Candida* spp. have not yet been determined. Susceptible isolates to anidulafungin could be considered susceptible to caspofungin too.

Note[2]: *Candida auris* breakpoints against different antifungals have not yet been defined by the CLSI and EUCAST methods.

SDD= Susceptible-dose dependent.

Candida glabrata

Candida glabrata is usually the second causative agent of invasive candidiasis and the first most common NAC-species causing BSI, occurring in approximately 29% of the patients (114–116). To date, several studies highlighted the abundance of *C. glabrata* among patients with hematologic malignancies, diabetes, and those with an abdominal source of infection, that could even surpass the number of *C. albicans* infection (34, 117–119). Like other yeasts, *C. glabrata* is an opportunistic pathogen and several virulence factors are involved in transforming this microorganism into its invasive form. Each factor can change the balance between *C. glabrata* and the human body leading to invasive candidiasis. Biofilm formation and expression of cell wall adhesion proteins are two major virulence factors involved in the pathogenicity of *C. glabrata*. Invasive *C. glabrata* infections are associated with biofilms formation in human host tissues and medical devices, like catheters, due to the presence of several adhesion proteins on

the cell wall (120, 121). *C. glabrata* Cell Wall Proteins (CWPs) are needed to produce biofilms, and so far, two groups of these proteins are known based on the form of binding to the cell wall: **a)** proteins that bind to β-1,3-glucan (PIR proteins) via a mild alkali-sensitive linkage, and **b)** proteins containing glycosylphosphatidylinositol (GPI) covalently bind to β-1,6-glucan via a modified anchor (120, 122). From 106 GPI-CWPs, 67 are identified as adhesion proteins based on their N-terminal substrate-binding domain (123). Among the adhesion proteins, Epithelial adhesion (Epa) and PA14 containing wall proteins (Pwps) are the first proteins involved in the formation of biofilms (123, 124). Besides its pathogenicity, resistance to different antifungals is another considerable concern regarding this yeast species.

An increasing number of *C. glabrata* isolates that are resistant to different antifungals has been reported from intensive care patients during the last two decades. *C. glabrata* is intrinsically less susceptible to commonly used antifungals, such as azoles (125). Fluconazole is considered as the first antifungal therapy for patients who either do not have severe invasive candidiasis or who did not formerly use fluconazole prophylaxis (116, 117, 126). Long-time exposure to azoles, e.g. by prophylaxis, is one of the primary causes of the reduced susceptibility of *C. glabrata* isolates to fluconazole and this may lead to resistance (34, 117). The expression and/or mutations of several genes, such as ABC transporter genes, pleiotropic Drug Resistance 1p (*PDR1*), *CDR1,* and *CDR2* can induce the resistance of the species to azoles. In contrast to *C. albicans* and *C. parapsilosis*, only a few studies addressed the effect of *ERG11* up-regulation in azole resistance of *C. glabrata* (127, 128). Efflux-mediated mechanisms, i.e. ATP-binding cassette (ABC) transporter genes, play a critical role in azole resistance in *C. glabrata*. Pleiotropic Drug Resistance 1p (*PDR1*) is a transcription factor encoded by the *PDR1* gene in yeasts. This protein is a key factor that regulates the expression of ABC-transporter genes, e.g. *CDR1*, *CDR2*, *PDH1*, and *SNQ2*, that are specifically involved in azole resistance. Mutations in *PDR1* are associated with multidrug-resistance in *C. glabrata* (126, 128–130). Ferrari *et al.*

(2009) revealed that mutations in *CDR1*, *PDH1*, *CDR2*, *SNQ2* genes plus *PDR1* not only impact the susceptibility to azoles, but also the virulence of *C. glabrata* (131).

The rising number of *C. glabrata* isolates resistant to azoles has led to the use of echinocandins as empirical treatment of invasive candidiasis caused by *C. glabrata*. Resistance to echinocandins emerged in the late 1990s and is expanding fast and has been reported from different areas worldwide (127, 130, 132). The prevalence of *C. glabrata* isolates that are resistant to echinocandins is documented in many studies and has been estimated to range between 3 and 15% of the isolates obtained from different geographical locales (133, 134). Furthermore, multidrug-resistant (MDR) *C. glabrata* are usually resistant to echinocandins (133–135). Resistance to echinocandins is due to substitutions of single amino acids in specific hot spot regions, namely HS1 and HS2 of *FKS1* and *FKS2* genes. So far, several mutations have been identified in both *FKS1* and *FKS2*, i.e. S629P, R665G, F625C, F659Y, R636S, R631G, I634V in *FKS1*, and S663P, S663F, S663Y, R665G, F659Y, P667H, D666Y in *FKS2*. Among all mutations, S629P in *FKS1* and *FKS2* is the most frequently detected among clinical isolates (57, 132, 136, 137). Few studies suggested that mutations in *FKS2* may occur due to pressure by the host that entails a compensatory reaction of *C. glabrata* (130, 138). Mutations in both or one of the hot spots of *FKS1* and *FKS2* genes lead to elevated MICs values for echinocandins using both the CLSI and EUCAST methods (56, 130, 139).

Resistance to AMB is not common among *C. glabrata* isolates. Decreasing susceptibility or resistance to AMB is caused by mutations in genes encoding sterol biosynthesis. The major mutations occur in *ERG2* and *ERG6* and impede ergosterol synthesis. A mutation in *ERG6* results in substituting of cysteine by phenylalanine (C198F) in the ergosterol synthesis and causes a deficiency of ergosterol of the cell membrane, which reduces the susceptibility of *C. glabrata* isolates to AMB and may result in resistance to AMB (125, 140, 141). Table 2

describes *C. glabrata* breakpoints for azoles and echinocandins based on the CLSI and EUCAST methods (112, 113).

Candida auris

The epidemiology of invasive candidiasis is changing in the last decades due to altering circumstances, such as improvement of diagnostic methods, an increase of the population of patients at risk, climate changes, and/or geographical and temperal aspects (142). *Candida auris* has recently emerged as a new NAC-species since its description about 10 years ago in Japan (143). The species was firstly isolated from the external human ear and turned into a serious invasive pathogen that is isolated from burn wounds, blood, urine, bronchoalveolar lavage (BAL), and tissues (144, 145). The species is reported to cause nosocomial outbreaks in different geographical regions, such as India, Colombia, Kuwait, the United Kingdom, the United States, and Venezuela (63, 146–149). *C. auris* can survive and colonize on a broad range of surfaces in the hospital environment and may remain alive up to 90 days *in vitro* based on different conditions, according to the USA Centers for Disease Control and Prevention (CDC) (150). This yeast has been collected from different objects and facilities that patients contact directly (i.e. chairs, bed trays, medical equipment, and mobile phones) or, indirectly, via their healthcare workers (i.e. closet cabinets, door handles, alcohol gel dispensers) (143). The species can be easily transferred between patients and hospital environments and this is one of the reasons for its prompt expansion on the globe. Like other yeasts, it is an opportunistic agent that can colonize the human body without any symptoms for several months (143, 150). For high-risk patients, invasive candidiasis caused by *C. auris* occurs in both sexes with a mortality range of 30 to 70% (143, 151, 152). *C. auris* pathogenicity relates to different virulence factors, including adherence, biofilm formation, and phospholipase and proteinase

production (64). These factors are, however, also known as virulence factors involved in the pathogenicity of other *Candida* species. Larkin *et al*. (2017) suggested that the activity of the respective factors may be different based on the clade where the *C. auris* isolates belong to. Interestingly, they showed that all these virulence factors are less active in *C. auris* compared to other *Candida* spp., particularly *C. albicans* and *C. parapsilosis* (64). For instance, two genes, candidalysin (*ECE1*) and hyphal cell wall protein (*HWP1*), that are responsible for biofilm formation in *C. albicans*, are not present in *C. auris* and closely related species. The absence of *HWP*1 might be a reason that *C. auris* biofilms are thinner than those of *C. albicans*. The production of lytic enzymes has been demonstrated in *C. auris*, but is strain-depended (153). So far, hydrolases, transferases, and oxidoreductases were found as the major enzymes involved in adherence and invasion of *C. auris* to host cells (153).

Whole-genome sequencing of *C. auris* showed a high similarity between this species and *C. haemulonii*, *C. duobushaemulonii*, and *C. pseudohaemulonii* (154, 155). This is not surprising as they all belong to the same clade within the Metschnikowiaceae family (143, 154–157). Due to this genetic and phylogenetic similarity, the identification of *C. auris* by conventional biochemical techniques and some commercial methods has been a challenge in routine clinical laboratories (158). *C. auris* has been misidentified as *C. haemulonii*, *C. famata*, *C. sake*, *C. catenulate*, *C. parapsilosis*, *Clavispora lusitaniae*, *Meyerozyma guilliermondii*, and *S. cerevisiae*, and even as basidiomycetous yeast species, such as *Rhodotorula glutinis* and *Rhodotorula mucilaginosa* (143, 158, 159). BioMerieux Company has updated its VITEK-2 system database in order to improve identification of *C. auris* isolates (143, 146). However, the most favored technique for its identification is Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS).

As early as 2012 Cendejas-Bueno *et al.* (2012) showed for the first time the identification of *C. auris* by MALDI-TOF MS, and the results were confirmed by sequence analysis of the

internal transcribed spacer (ITS) regions of the nuclear rRNA gene operon and AFLP (154). In 2019, the Bruker MBT Compass Library, Revision E MBT 7854 was updated and an USA version of the respective library was cleared by the FDA to be used in clinical laboratories for *C. auris* identification. By this method, *C. auris* can be correctly and rapidly identified using cultures on Sabouraud dextrose agar (SDA) and blood cultures (160).

Resistance to at least one antifungal drug, mainly fluconazole, occurs in *C. auris* isolates. However, multidrug-resistant isolates (MDR) are on the rise. So far, only 3% of isolates are resistant to all three classes of antifungals, and 41% of isolates are resistant to two drug classes (161, 162). Lockhart *et al.*, 2017 identified F126T, Y132F and K143R mutations in *ERG11* of *C. auris* isolates that are resistant to azoles (151). Like in *C. albicans*, several studies demonstrated that efflux genes play a key role in the MDR mechanism to azoles, polyenes, and echinocandins (151, 161, 162). So far, echinocandins have been suggested as a first-line antifungal therapy against *C. auris* infections (60, 163). However, intensive application of this antifungal has caused the emergence of isolates that are resistant to echinocandins (60, 163). Chowdhary *et al.* (2018) reported *C. auris* isolates resistant to echinocandins that harbored an S639F mutation in the *FKS1* gene (164). Neither the CLSI nor EUCAST methods have released a fixed breakpoint for *C. auris* against different antifungals. Therefore, the applicability of antifungal susceptibility testing of *C. auris* isolates is still under debate in research and clinical laboratories (Table 2) (160, 165, 166).

Typing of *C. auris* isolates got recently considerable attention. Molecular analysis based on the ITS regions sequencing, whole-genome sequencing (WGS) and differentiation of N-acetyl glucosamine (NAG) utilization between isolates were used to distinguish between isolates from different geographic regions (146, 151, 167, 168). Recently, there is growing evidence on the importance of recognizing genotypes of *C. auris* to understand its epidemiology. Accordingly, genotyping studies used whole-genome sequencing (WGS) (168), sequencing of the ITS

regions, and the D1/D2 domains of the large subunit ribosomal DNA, *RPB1* and *RPB2* genes (146), and amplified fragment length polymorphism (AFLP) analysis (146, 169). Data from a phylogenomic analysis classified *C. auris* isolates into four clades, and a new study identified a fifth clade in Iran (170). Isolates from each clade have a distinct phylogenetic structure with significant genomic diversity among the clades that ranges from ten to thousands of SNPs between the Southern Asian (India, Oman, Pakistan), Eastern Asian (Japan, Korea), South African, and South American (Colombia, Venezuela) populations (146, 151, 167, 168). So far, several *C. auris* nosocomial outbreaks have been reported worldwide, and therefore, it is relevant to study the characteristic of how this yeast can be transfered from the environment to a human`s body. The first nosocomial outbreak was reported from South Korea (62), followed by India (171, 172), South Africa (173), Kuwait (148), Venezuela (174), the USA (175), and Europe (176, 177). The development and implementation of methods to monitor *C. auris* outbreaks in the hospitals are important to control the transfer of this pathogenic microorganism between patients, hospitals healthcare workers, medical devices and other materials, and the environment to subsequently reach proper management of the patients.

Antifungals, drug resistance and antifungal susceptibility testing

Rapid and accurate identification of *Candida* species is an urgent demand for epidemiological studies, and most important for proper patient management. To select a proper antifungal agent against invasive candidiasis, it is important to identify the infectious agent at the species level (178). Accordingly, many laboratories are equipped with automated or semi-automated diagnostic systems. Concomitantly, new, accurate and rapid antifungal susceptibility methods are being developed.

Furthermore, many efforts recently focused on developing new antifungal drugs. Therefore, combining the application of an appropriate diagnostic method, treatment with an antifungal

agent, and the rapid and accurate antifungal susceptibility assessment to antifungals may result in a reduction of the high mortality rate of yeast-related infections (123, 124).

Due to the growing challenge of fungal diseases, several antifungals have been developed. The first antifungal, griseofulvin, was isolated from the mold *Penicillium griseofulvum* in 1939, but it was not introduced to the clinical until 1958 when its efficacy against fungal infections was shown (180). Presently, there are five main classes of antifungals used against human fungal diseases: 1) polyenes, 2) pyrimidine analogs, 3) azoles, 4) echinocandins, and 5) allylamines, thiocarbamates, and morpholines (181–183). Polyenes are the oldest antifungal made in the 1950s, followed by azole derivatives in the 1960s (180, 184). Echinocandins were discovered and introduced to the market in the 1970s, but only approved by the European Agency for the Evaluation of Medicinal Products (EMEA) and the Food and Drug Administration (FDA) in the 1990s. Among echinocandins, anidulafungin is the most recently developed antifungal and allowed for use in the clinic in 2006 (180, 185). Antifungal agents are administrated differently, namely orally, topically and intravenously. A few antifungals are frequently prescribed to treat invasive fungal infections, the number of which is rising and increasingly isolates are resistant to antifungals. Therefore, there is a need to discover novel antifungals. New antifungals are mostly found by studies and analysis of naturally occurring or synthetic/semisynthetic chemical compounds (181, 185). A couple of limitations hamper the development of new antifungals. For instance, the main fundamental challenge is that the fungi are phylogenetically closely related to their animal/human hosts, and both are eukaryotic organisms. Consequently, many similar biochemical and cell biological processes occur in both fungi and humans, and many toxic molecules to fungi are also harmful to human cells. Due to these similarities between fungi and human/animals, many newly developed antifungal agents do not pass clinical trials (186, 187). The next principal to develop new antifungals is finding a specific target that is not available in the human host. For instance, the fungal cell wall is a main target

for antifungals because of its unique structure which is absent in human cells. Only a few cell wall/membrane–targeted molecules and/or their biosynthesis have been discovered so far. The main mechanisms of action of antifungals targeting the cell wall are focused on the inhibition of chitin and β-glucan synthesis. For instance, glycosylphosphatidylinositol (GPI) biosynthesis, β-1,3-glucan synthesis by blocking the enzyme glucan synthase, and β-1,6-glucan synthesis are several of the cell wall-related drug targets and biosynthetic routes that are inhibited by different antifungals. A couple of antifungals bind to the cell membrane ergosterol and/or inhibit ergosterol biosynthesis via the cytochrome P450 enzyme, 14-α demethylase, that catalyzes the conversion of lanosterol to ergosterol. This function makes pores in the cell membrane and leads to cell death. Another mechanism is impairing cell membrane permeability by impacting on a cascade of oxidation reactions and interactions with lipoproteins in the cell membrane that causes the release of free radicals (186, 188, 189). Furthermore, other aspects like unwanted side effects, range of activity, pharmacokinetics activity, and safety profiles must be considered when developing a new antifungal (181, 186). Antifungal treatment failure is relevant to either/both the host (clinical resistance) or/and fungi (microbiological resistance) (181, 190). Clinical resistance refers to the lack of a proper response of the human body to the administered dose of the respective antifungal. Different factors may influence developing clinical resistance. The host immune system is the main factor playing a synergistical role with antifungals to eliminate the infection. Therefore, immune dysfunction may lead to failing therapy, because the antifungal agent must remove the fungus without the support of the immune system. Other factors, including antifungal pharmacokinetics/pharmacodynamic activity, antifungal concentration, the way of drug administration, penetration of the drug, and distribution through the host´s body play a role in the effectiveness of an antifungal agent. In general, pharmacokinetics (PK) refers to the concentration-time courses of the drug in the host body, and pharmacodynamics (PD) describes

the effect of the respective concentration of the drug (191, 192). Moreover, applying surgical devices and catheters improve biofilm formation by attaching microorganisms onto the devices and these biofilms are less susceptibility to antifungals (190, 193).

Microbiological resistance refers to a reduction of the susceptibility of a fungal isolate to an antifungal agent. This type of resistance can be classified into primary resistance (intrinsic/inherent resistance) and acquired resistance (extrinsic/acquired resistance) (181, 194). The number of fungi that are intrinsically resistant to the variant classes of antifungals is increasing and becoming a critical issue in the clinical practice (194, 195). Inherent resistance relates to microorganisms that are inherently resistant or less susceptible to an antifungal upon appropriate antifungal therapy. This type of resistance happens naturally among some fungi without being exposed to the antifungals (196). A typical example of this are *Cryptococcus* species that are intrinsically resistant to echinocandins (196). Other examples are *Pichia kudriavzevii* (= *Candida krusei*) and *C. glabrata* that are resistant (and/or less susceptible) to fluconazole, and *Clavispora lusitaniae* that is resistant to amphotericin B (197). Acquired resistance is usually detected among fungi which are important in the clinic. These fungi were susceptible before being exposed to antifungals, and resistance develops upon prolonged exposure to an antifungal and is usually related to mutations that alter gene expression. Several molecular mechanisms, such as drug target alternation and/or overexpression, upregulation of multidrug transporters and activation of stress responses, are involved in the development of acquired antifungal resistance (181, 194, 198). Below we describe the five main classes of available antifungals and some new drugs that are in development. Subsequently, resistance to antifungals is described more in detail.

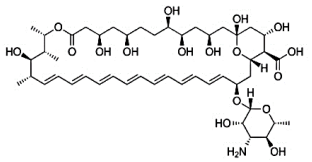
Polyenes

These components are cyclic amphiphilic organic molecules produced by *Streptomyces* species. As Figure 2 shows, polyenes include of a large ring of atoms which is surrounded by carbon-carbon double bonds on the hydrophobic side and multiple hydroxyl residues at the hydrophilic side, conjugated with a d-mycosamine group (Figure 2a).

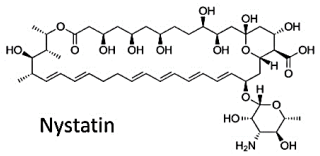
Ergosterol is the main sterol of the fungal cell membrane which is targeted by polyenes. Polyenes bind to the ergosterol via their amphiphilic structure and make pores in the fungal cell membrane. The formation of porin channels causes instability of the plasma membrane. Subsequently, intracellular components, such as K+ ions, are leaking outside and this process leads to cell lysis (199). Additionally, some studies demonstrated that another function of this antifungal class is to induce oxidative stress in some *Candida* spp., including *C. albicans* (199, 200). Amphotericin B (AMB), nystatin and natamycin are the most common polyenes used against fungal infections. Amphotericin B is the first candidate against invasive candidiasis targeting ergosterol. This antifungal has a high hydrophobicity and less absorption through the gastrointestinal tract. Therefore, AMB is applied intravenously in contrast to nystatin and natamycin that are usually used topically or orally, (182, 199). However, AMB has a strong negative effect on the kidney and liver. The toxicity and side effects, e.g. nephrotoxicity, are a major concern related to the use of these antifungals, rather than resistance (199, 201). Accordingly, several efforts have been made to minimize this issue, and this resulted in new formulations of this antifungal, such as liposomal AMB or lipid AMB complexes (199). Another limitation of these antifungals is associated with their slight affinity to the cholesterol present in human cells.

a.

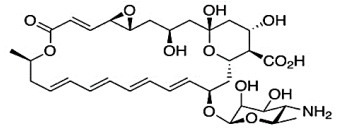
Amphotericin B



Nystatin

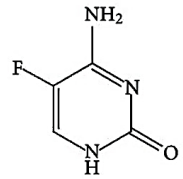


Natamycin

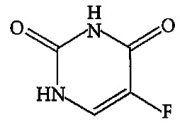


b.

5-fluorocytosine

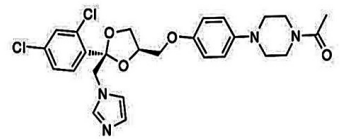


5-fluorouracil

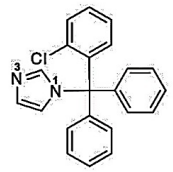


c.

Ketoconazole



Clotrimazole



Fluconazole

Voriconazole

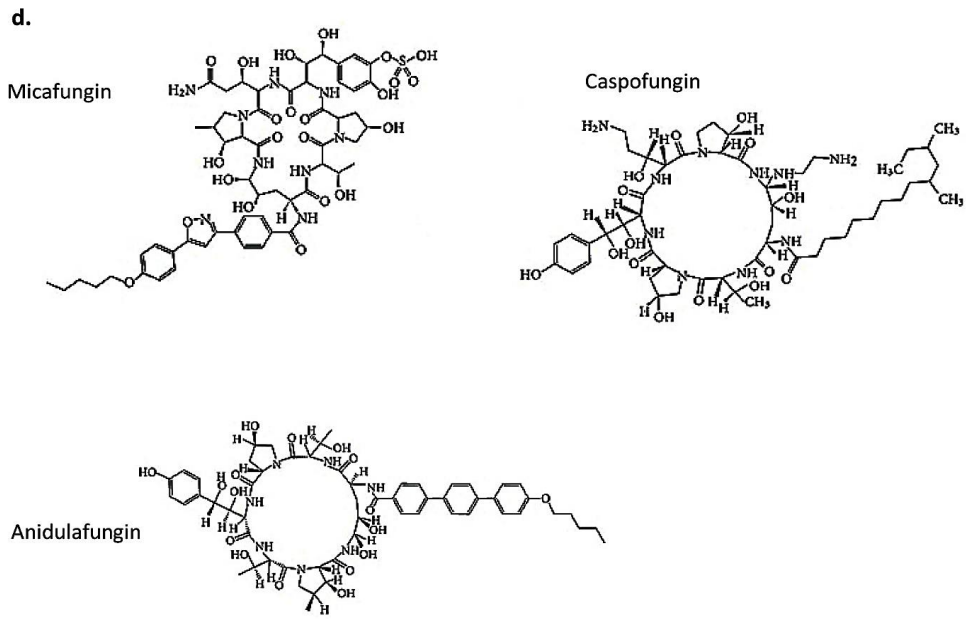


Figure 2. Chemical structure of the four main classes of antifungals. (a) Polyenes include amphotericin B, nystatin, and natamycin. (b) Fluoropyrimidines include 5-fluorocytosine and 5-fluorouracil. (c) Azoles include two examples of imidazoles and two examples of triazoles. (d) Echinocandins include micafungin, caspofungin, and anidulafungin. These figures are taken from Vandeputte P *et al.* (2012) [199].

Resistance to polyenes

Polyenes, particularly AMB, have been used as an efficient antifungal for more than 40 years with only few reports of acquired or innate resistance to the drug. Resistance to AMB is not frequently detected in clinical isolates; however, there are several reports of increasing MICs to AMB in *Pichia kudriavzevii* (= *C. krusei*) and *C. glabrata* isolates (202). *Clavispora lusitaniae* (= *C. lusitaniae*) (203) and *Trichosporon beigelii* (204) are two species that are less or not susceptible to polyenes. Filamentous fungi show more often than yeasts increased MICs

to polyenes (194). Resistance to polyenes is associated with a mutation in the *ERG* genes that causes alterations in the ergosterol synthesis pathway and accumulation of sterol intermediates (Figure 1). Mutations of the *ERG3* and *ERG11* genes leads to a reduced ergosterol content in the cell membrane and increased MICs. Moreover, not only a reduction in the ergosterol content, but also changes in the cell wall can cause resistance to AMB (201, 205, 206).

Pyrimidine analogs (Fluoropyrimidines)

Fluoropyrimidines are synthetic structural analogs of the DNA nucleotide cytosine. These antifungals are administrated orally and intravenously. Due to their small size and high hydrosolubility, they can distribute rapidly through the human body (199). Fluoropyrimidines enter the fungal cell via cytosine permease and interact with RNA via 5-fluorouridine-triphosphate that leads to inhibition of the production of certain proteins, such as cytosine deaminase in *C. albicans* (*FCA1*) and *C. glabrata* (*FCY1*) (207). Furthermore, they also cause DNA damage by inhibiting the thymidylate-synthase enzyme (199, 208, 209). Two antifungals belonging to this class are being used against fungal human diseases, namely 5-fluorocytosine (5-FC) and 5-fluorouracil (5-FU) (Figure 2b). Fungi may rapidly develop resistance to these when administered as monotherapy. A high number of reports on innate or acquired resistance to this class of antifungals made that they are usually applied in the clinic in combination with AMB and/or azole antifungals, such as fluconazole or itraconazole (199). The advantages of these antifungals are the broad spectrum activity against several microorganisms, including fungi like *Candida*, *Cryptococcus*, *Phialophora*, *Cladosporium,* and *Aspergillus* species, and some protozoa as well. Additionally, so far, no significant side effects have been reported, but they may cause some adverse effects like hepatotoxicity or bone marrow lesions (182, 199, 210).

Azoles

Azoles include several antifungals against fungal infections that are widely used in the clinic (199). These antifungals are cyclic organic molecules with a different number of nitrogen atoms in their structure for which they can be divided into two groups: 1) Imidazoles with two nitrogen atoms, including antifungals such as ketoconazole, miconazole, and clotrimazole. This group is exclusively used for systematic candidiasis; 2) Triazoles with three nitrogen atoms, including fluconazole, voriconazole, itraconazole, and posaconazole. Fluconazole is widely used against yeast infections, but, voriconazole, itraconazole, and posaconazole are also used to treat infectious by filamentous fungi (Figure 2c) (182, 199).

To date, several studies focused on their function, pharmacological properties, and the mechanism of microorganisms to develop resistance. Azoles have fungistatic properties, and their negative interference with cell growth and proliferation cause fungal cell death (209). They inhibit lanosterol 14-α demethylase by binding to the iron atom of the heme group of this enzyme via their free nitrogen atom. This enzyme is encoded by the *ERG11* gene and plays a key role in the biosynthesis of ergosterol on the fungal cell membrane (199, 211, 212).

Ketoconazole has been used for the treatment of systemic fungal infections. Due to its hydrophobic structure, it has not been used intravenously, and it is poorly absorbed orally. Additionally, this drug has a number of limitations when administered, such as reduced activity in immunosuppressed patients, a negative effect on the production of testosterone or glucocorticoids, development of liver and gastrointestinal complications, and cross-reaction with other drugs (199, 213). To overcome these limitations other azoles were developed, including triazoles (199). Fluconazole, as one of the most commonly known triazoles, has a hydrosoluble structure and is usually administered intravenously. It is frequently used against superficial candidiasis, but due to the increasing number of fungal infections, the prescription

of fluconazole has also expanded to treat disseminated candidiasis, cryptococcal meningitis, and cutaneous candidiasis. The wide use of fluconazole led to the rise of numerous fungi that are resistant to this antifungal (199). Furthermore, fluconazole has a cross-reaction to drugs used for chemotherapy and AIDS treatment (214). Hence, a new generation of triazoles was developed. Voriconazole and posaconazole are two newly developed antifungals that belong to the triazole group. The advantage of these antifungals is a broad spectrum of activity against a wide range of fungi compared to fluconazole and ketoconazole. For instance, they are also effective against species belonging to the genera *Aspergillus* and *Sporothrix* (215). Compared to ketoconazole, they are less toxic, but they have the same side effects and drug interactions as fluconazole. Furthermore, most of the fungal isolates that are resistant to fluconazole are also resistant to voriconazole, posaconazole, and itraconazole (172,179).

Resistance to azoles

The azole antifungal class is the most commonly used class of antifungals in clinical laboratories and the majority of drug-resistant *Candida* spp. relate to this drug class. Four main molecular mechanisms may cause azole resistance: 1) Target mutations, 2) Target expression deregulation, 3) Alternation in ergosterol biosynthesis enzymes, and 4) Increase of drug efflux pumps (Figure 1) (194).

Target mutations refer to point mutations in the *ERG11* gene, which encodes the enzyme lanosterol 14-α-demethylase in yeasts. This enzyme is involved in the conversion of lanosterol to other sterols such as ergosterol as one of the main sterols occurring in the fungal cell membrane. Mutations of lanosterol 14-α-demethylase cause a reduction or inhibition of the binding of azole to the enzyme (181, 217). Target expression deregulation is a feedback mechanism, in which *ERG11* gene upregulation prohibits saturation with azoles (209). In *C.*

albicans, overexpression of the *ERG11* gene is due to a point mutation in the transcriptional activator Upc2 (194, 218). So far, the mutation caused by the target expression deregulation has been observed in *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *Pichia kudriavzevii* (= *C. krusei*) (190). The mutations in the *ERG11* gene are not the only reason for an alteration in ergosterol biosynthesis enzymes, but it may also be relevant to other enzymes of the same biosynthetic pathway. For instance, a mutation in the *ERG3* gene that encodes sterol Δ5,6-desaturase results in accumulation of 14-α-methyl-3,6-diol in the fungal cell membrane causing alteration of the cell membrane composition. 14-α-methyl-3,6-diol is an alternative sterol that changes cell membrane function and contributes to increasing resistance to azoles. Moreover, *ERG3* mutations provoke increase of the expression of stress molecules, like calcineurin, a calcium-dependent serine/threonine protein phosphatase, protein kinase Pkc1, and the molecular chaperone Hsp90 in the cell membrane, resulting in increased tolerance of the above mentioned *Candida* species to azoles (219, 220). Additionally, it has been observed that a mutation in the *ERG3* gene leads to cross-resistance between azoles and polyenes (181, 194, 198). Another possibility to develop azole-resistant *Candida* spp. are mutations in the genes encoding efflux pumps that cause the induction of those multi-drug pumps. This function reduces the concentration of azoles inside fungal cells (221). Two different drug efflux systems remove azoles from the cytoplasm in fungi: 1) *CDR1*- and *CDR2*-type pumps which are ATP-binding cassette (ABC) transporters superfamily, and 2) Mdr1 which is a major facilitator superfamily (MFS)-type pump. Overexpression of the genes producing ABC proteins leads to azole resistance in some *Candida* species (207, 222). In *C. albicans CDR1* and *CDR2* genes, and in *C. glabrata,* the *CgCDR1*, *CgCDR2*, and *CgSNQ2* genes encode ABC proteins in the cell membrane. Mutations of the respective genes lead to development of resistance to azoles (199, 207). Among the multiple genes producing 95 MFS proteins in *C. albicans*, the *CaMDR1p* gene is responsible to remove drugs from the cytoplasm. A couple of studies have

shown a link between overexpression of the *CaMDR1p* gene and resistance to azoles (190, 223).

Echinocandins

Echinocandins are a recently developed class of antifungals that are approved by the Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medicinal Products (EMEA). This class includes caspofungin (2001-2002), micafungin (2005), and the latest one is anidulafungin (2006) (199, 224). Echinocandins are all water-soluble and administered intravenously. They have shown fungicidal activities against all *Candida* species (225). Echinocandins are synthetic derivatives of lipopeptides with a similar cyclic hexapeptide that is linked to a long modified N-linked acyle fatty acid chain (Figure 2d) (226). Echinocandins act by binding directly to the glucan synthesis catalytic subunit and inhibit the β-1,3 glucan synthase enzyme. This enzyme is encoded by *FKS* genes and produces β-glucan as the most prevalent component of the fungal cell wall. Inhibition of β-1,3 glucan biosynthesis leads to the change of cell wall structure and makes it weaker and unstable to osmotic stress (209). Since animal cells do not have β-1,3 glucan and this carbohydrate polymer is present only in the fungal cell wall, it is a suitable target for antifungals such as echinocandins (182, 227, 228). Echinocandins inhibit a wide spectrum of fungal species, including *Candida* and *Aspergillus* species, but they are less efficient against *C. parapsilosis* and *C. guilliermondii* (101, 103). Because the cell wall of Zygomycetes, Basidiomycetes such as *Cryptococcus neoformans,* and *Fusarium* species contains less β-glucans, echinocandins are not active at clinically relevant concentrations against these fungi (103, 182, 229, 230). Due to their unique target that is only available in the fungal cell wall, side effects or cross-reaction of these antifungals in the human body have not been reported. Echinocandins are recommended antifungals in case of infection due to *Aspergillus* and *Candida* species that are resistant to

azoles or polyenes. Therefore, if one of the *Candida* species shows resistance to azoles, caspofungin is the first-line therapy for fungal prophylaxis, and to treat invasive candidiasis and aspergillosis. Almost 70% of patients refractory to other antifungals are responding to caspofungin (199, 209). Since the administration of caspofungin has no significant adverse effects on the liver and kidney, it is used widely *in vivo* against fungal infections (199, 231). *In vitro* antifungal susceptibility testing of caspofungin is not recommended due to the paradoxical effect that has been observed for some *Candida* species, such as *C. albicans* and *C. glabrata* (231). Micafungin is highly effective for treatment of esophageal candidosis, and it is usually prescribed for prophylaxis in bone-marrow transplant patients. Micafungin has no side effects in patients with renal and/or hepatic impairment. Furthermore, both adult and pediatric patients tolerate this antifungal drug well. Therefore, micafungin can be widely used against invasive candidiasis in all types of patients (209, 232, 233). Anidulafungin is commonly administered for esophageal and/or invasive candidiasis. This drug spreads rapidly over the body without adverse effects to the liver and kidney, and, therefore, it is recommended for use in patients with liver and/or kidney failure (199, 234). Several advantages of anidulafungin, for instance, a proper pharmacokinetics and pharmacodynamics profile, rapid distribution in the body (1h) which is significantly shorter than that of caspofungin (9 to 11h) and micafungin (14h), and less side effects, have turned it into one of the favorable antifungals used in a wide spectrum of patient applications. Contrary to caspofungin, the paradoxical effect has not been observed for anidulafungin and micafungin in *in vitro* antifungal susceptibility tests (235).

Rezafungin (CD101) is a new echinocandin compound that is under phase II evaluation in clinical trials for invasive candidiasis. It is administered intravenously once per week to treat and prevent invasive fungal infections. Rezafungin has a high plasma drug exposure compared to other echinocandins drugs that is related to its high level of stability and pharmacokinetic/pharmacodynamic activity. Several studies aimed to evaluate the antifungal

activity of this novel drug for different fungal species (236–238). In one study, Arendrup *et al*. (2018) studied the pharmacokinetics activity of rezafungin against the most prevalent clinical pathogenic *Candida* species. Their findings indicated that *C. albicans* has the lowest MIC to this antifungal, whereas, *C. parapsilosis* had the highest MIC. Overall, this antifungal successfully inhibited the growth of *Candida* species at different concentrations (236). More recently, the activity of rezafungin against *C. auris* was analyzed and compared to amphotericin B and micafungin *in vitro* and *in vivo* in mice. The findings of this study showed an equal antifungal activity of rezafungin with a significantly lower average log10 cfu/g of tissue compared to amphotericin B on all days and compared to micafungin on day 10 when the kidney was harvested. Rezafungin has an analogous structure to anidulafungin with an extra choline moiety at the C5 ornithine position. This unique structure of rezafungin increases the chemical stability to host degradation pathways and may cause it difficult to develop resistance to this drug (239, 240).

Resistance to echinocandins

The prevalence of fungal species that are resistant to echinocandins, is still less than other antifungals. The first report for *Candida* species resistant to echinocandins was published in 2005 (241). Although the prevalence of *C. albicans* and other *Candida* species that are resistant to echinocandins has recently increased, it is still relatively less with about <3% of total antifungal-resistant *Candida* isolates (242).

Resistance to echinocandins develops via amino acid substitutions within highly conserved hotspots (HS) of the *FKS* genes. In *C. albicans* and most other *Candida* species echinocandin resistance develops via point mutations in two different hotspots (HS1 and HS2) of the *FKS1* gene (190). The most common mutations have been found in amino acid residues Ser645 and

Phe641 in *FKS1*. Other detected amino acid substitutions are Phe641, Pro649, and Arg1361 in the *FKS1* gene. In *C. glabrata*, point mutations in the HS1 and HS2 of the *FKS1* and *FKS2* caused the development of resistant isolates. In *C. glabrata*, the mutations mostly occur in the *FKS2* gene (190). Substitution in the *FKS2* Ser663 is the most prevalent mutation causing the highest resistance in this species. This mutation is equivalent to *FKS1* Ser645 in *C. albicans* (Figure 1) (210, 243, 244). In contrast, in basidiomycetous yeast species like *Cryptococcus neoformans* resistance to echinocandins is not caused by a mutation in *FKS* genes, but is probably due to a different polysaccharide composition of the cell wall of this and other basidiomycetous species (196, 199).

Furthermore, other factors next to gene mutations can develop resistance or poor tolerance to echinocandins. With respect to an adaptive response mechanism to environmental stresses, cell wall components have a crucial role to mediate signaling causing tolerance and resistance to echinocandins. For instance, in the presence of echinocandins, different proteins and enzymes like Protein Kinase C (PKC), the protein phosphatase calcineurin, and the molecular chaperone Hsp 90 are activated and inhibit β-glucan synthesis. Subsequently, β-glucan synthesis suppression causes depletion or reduction of the β-glucan content in the cell wall (245–247). Another cell wall action promoting echinocandin resistance is a compensatory mechanism that results in increased chitin synthesis. Chitin and β-1,3 glucan are two major components of the yeast cell wall, and upregulation of chitin synthesis changes the balance of the cell wall structure. Strains with higher chitin in their cell wall show less susceptibility to echinocandins (135, 138, 241, 248). An elevated chitin content results in paradoxical effects of mutant cell walls and leads to cell growth at higher concentration of the antifungals (246, 249).

Echinocandins resistance in clinical practice

The *FKS* genes mutations do not commonly occur among all clinical yeast isolates. Mutations occur predominantly in *C. albicans* and *C. glabrata* after long term administration of echinocandins (247). Echinocandins are the preferred antifungal drugs to treat patients with invasive candidiasis, and, therefore, in terms of lower susceptibility or resistance of *Candida* yeasts to this class of antifungals, susceptibility testing or *FKS* genotyping, or both are strongly recommended. *FKS* genotyping, however, is not commonly done in the clinical laboratories, and, therefore, the CLSI or EUCAST methods to perform the antifungal susceptibility test (AFST) are frequently used. To define the MIC for every *Candida* species against different agents, intensive epidemiological studies and surveillance for resistance are required. *In vitro* standard microdilution methods have not yet defined a confident breakpoint for caspofungin, and it is therefore recommended to use anidulafungin and micafungin instead (241, 247).

Allylamines, thiocarbamates and morpholines

This class of antifungals has a wide spectrum of activity against different fungal species. Due to their adverse effects, such as gastrointestinal symptoms, rash, urticaria, and pruritus, they are usually limited to treat dermatophyte infections, including *tinea capitis*, *tinea pedis*, and onychomycosis (182). These antifungals are usually used topically, while the allylamines can be administered orally for systemic absorption. They have lipophilic and keratophilic activities and accumulate in the stratum corneum of skin and nails., and, therefore, they are considered to treat skin and nails infections caused by dermatophytes (250). This class of antifungals is involved in inhibiting the production of different enzymes that are involved in ergosterol biosynthesis. The allylamines (terbinafine) and thiocarbamates (tolnaftate) inhibit the enzyme squalene epoxidase that is encoded by the *ERG1* gene and is involved in an early step of the

ergosterol biosynthesis pathway. The morpholines (fenpropimorph) inhibit two other enzymes, namely C14-reductase encoded by *ERG2*, and Δ7,8-isomerase encoded by *ERG24*, which both are involved in ergosterol biosynthesis (Figure 1) (182, 199, 251). Fenpropimorph has a limited application since it can inhibit mammalian and higher-plants sterol biosynthesis by affecting lanosterol demethylation and the cycloeucalenol-obtusifoliol isomerase, respectively (250).

MYC-053

MYC-053 [sodium 5- [1- (3,5-dichloro-2- hydroxyphenyl) methylideneamino]-6-methyl-1,2,3,4- tetrahydro-2,4-pyrimidinedionate] is a new chemical class of antifungal compounds that has no chemical and structural relation to any of the other available antifungals. MYC-053 is used orally and intravenously and inhibits nucleic acid and chitin biosynthesis in fungi. Inhibition of chitin synthesis changes the balance of the cell wall components, and prohibition of nucleic acid synthesis inhibits cell proliferation that both lead to killing of cells (252). MYC-053 is often used for fungal infections caused by agents showing cross-resistance, like *C. glabrata*, or multidrug-resistant species, such as *C. auris*. *In vitro*, MYC-053 antifungal activities have been shown against several yeasts and yeast-like pathogens, such as species of *Candida*, *Cryptococcus*, and *Pneumocystis* (252, 253).

Multidrug-resistant *Candida* species

More recently, attention has focused on the emerging clinical multidrug-resistant (MDR) *Candida* species. The widespread use of different antifungals during prophylaxis, and empirical and targeted therapy of fungal infections in different patient groups resulted in the emergence of MDR yeasts (254, 255). The parallel activation of different molecular mechanisms, adaptive responses, signaling and stress response pathways contributed to the development of multidrug-resistant of isolates belonging to *Candida* species (201). *C. glabrata* and *C. auris*

are two of the most common MDR NAC species (171, 256). The majority of the *C. glabrata* isolates are inherently resistant to azoles. Simultaneously, the number of *C. glabrata* isolates resistant to echinocandins has been rising, thus resulting in a significant increase of MDR *C. glabrata* isolates (194). The emergence of hypermutator lineages is one of the mechanisms that led to the development of MDR isolates (257). In one study, 1300 C. *glabrata* isolates were tested and one-third of them were resistant to both echinocandins and azoles (258). This was due to a hypermutator phenotype and mutations in the DNA repair gene *MSH2* that confer isolates to become resistant to multiple antifungals. It is estimated that 55% of clinical MDR isolates carry these mutations (258). Patients infected with invasive candidiasis caused by MDR *C. glabrata* should be treated with amphotericin B (258, 259). In *C. albicans* isolates that are resistant to both echinocandins and azoles overexpress both *CDR1* and *CDR2* (260). *C. auris* is the first emerging yeast pathogen for which a majority of the isolates are inherently multidrug-resistant. Most of the of *C. auris* isolates are resistant to fluconazole (>60–80%), and 10-30% of isolates are resistant to amphotericin B. The molecular mechanism responsible for azole resistance in this species is yet unknown, but mutations in the *ERG11* gene and the genes encoding efflux pumps may be involved in developing resistant isolates (61, 65). Echinocandins are empirically used to cure *C. auris* infections. More recently, isolates that are resistant to echinocandins have been reported in India and had an S639F mutation in the *FKS1* gene (Figure 1) (61, 160, 164).

Antifungal susceptibility testing (AFST)

Detection of antifungal-resistant isolates by an accurate, reproducible and predictive antifungal susceptibility testing method is needed to decide on and start appropriate antifungal therapy. In the late 1980s and early 1990s, many efforts were spent on the development of susceptibility testing methods. Susceptibility testing of yeast and filamentous fungal species is still a

challenge in clinical diagnosis. The Clinical and Laboratory Standards Institute (CLSI, www.clsi.org) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST, www.eucast.org) are two reference methods widely used in clinical laboratories. Besides these two gold standard methods, there are some commercial approaches that are also used in clinical laboratories. Among the commercial methods, the epsilonmeter test (Etest) is the most popular technique in use in clinical laboratories. In the following, we will briefly outline all available methods that are used for antifungal susceptibility testing.

Broth-based microdilution methods

The Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methods are used as the reference methods in clinical laboratories to perform antibiotic/antifungal susceptibility testing (AST/AFST). Both are based on the broth microdilution method (BMD) and culture dependent. They are based on the preparation of a liquid broth medium named Roswell Park Memorial Institute Medium (RPMI 1640) including different concentrations of antibiotic/antifungal agents. A defined inoculum of microorganisms is added and the plates are incubated to analyze the growth of microorganisms (112, 113). These methods are broadly used for AFST of yeasts, such as *Candida* species, *Cryptococcus neoformans*, and filamentous fungi (261, 262). The procedure of sample preparation and the MIC interpretation are almost identical for both methods; however, they have slight methodological differences. The medium used, cell inoculation size, the shape of the microtiter wells, interpretation of the cell growth and in some cases, the MIC endpoints differ between the two methods (Table 3) (263). Both methods serve to define the MIC values of the respective fungal isolates using different concentrations of antifungals compared to growth in the control without antifungals. According to the fungal species, different incubation times within a range of 24 to 48 h are used (264, 265). The CLSI method

has recently established breakpoints for echinocandins, namely anidulafungin and micafungin, but not for caspofungin. The two methods have identical breakpoints for several *Candida* species against azoles and echinocandins (263, 264). These two methods do not properly work for all fungi when testing different antifungal agents. For instance, although caspofungin is the recommended antifungal prescribed to patients, neither the CLSI nor the EUCAST methods recommend *in vitro* caspofungin susceptibility testing. Testing the reproducibility of caspofungin testing has shown unreliable results; additionally, the paradoxical effect is observed in some *Candida* species, such as *C. albicans* and *C. auris* (160, 266). Hence, several efforts focused on the development of other AFST assays in commercial use.

Commercial AFST Methods

Disc diffusion and Epsilonmeter test (Etest)

Disc diffusion and Etest are two agar-based methods used for antifungal susceptibility testing of yeasts and filamentous fungi. With the disc diffusion method, MIC values are defined by the zone of growth inhibition around the paper disc containing the antifungal drugs. A known concentration of antifungal is employed in the disc to evaluate the growth after the incubation time. This method is widely used in routine clinical laboratories (263, 267).

Etest is another commercial method that is commonly used for *in vitro* AFST in research and clinical laboratories. BioMérieux (France), Liofilchem (Italy), and AB Biodisk (Sweden) are companies that produce Etest strips. The test can be done manually or automated by Simplex C76 (BioMérieux). The Simplex C76 is an automated device to make easy performing 1 to 6 different Etest strips at once (261, 268, 269). In this method, microorganisms are inoculated on RPMI agar medium (Roswell Park Memorial Institute) on which a plastic strip covered with a gradient of antifungals is placed. The MICs of *Candida* and mold isolates are determined by a

halo of growth inhibition around the strip occuring at a certain concentration of the drug after the appropriate incubation time. This method is reliable, has a high reproducibility and results largely agree with those obtained by the microdilution methods (268, 269). However, the MICs acquired for amphotericin B by Etest are usually higher than the concentration range obtained by the reference microdilution methods (263, 267–269). In addition, for assessing MICs of fluconazole of *C. tropicalis* and *C. glabrata*, Etest showed less agreement with results obtained by the reference microdilution methods, and this may be due to heavy trailing by strains of these species (270, 271). The trailing effect explains the reduced, but ongoing growth of yeasts in the presence of higher antifungal concentrations. The cause of this effect is not yet completely clear, but several studies have suggested different explainations. For instance, up-regulation of different genes encoding components involved in azole-resistance, such as *ERG1* (squalene epoxidase), *ERG11* (lanosterol demethylase), and efflux transporters, or the size of the inoculum and the incubation temperature may all contribute to the trailing effect. Adding extra glucose into the RPMI medium or decrease the pH to 5 may prevent this phenomenon (272). Both disc diffusion and Etest are easy to use and affordable as they do not need any special equipment. They need an incubation time of 24h or 48h, and when exposed to caspofungin, the paradoxical growth effect may happen to some *Candida* species (273). There are a couple of disadvantages of these methods, such as which medium to be used and the depth and moisture content of the plate agar. Preparing an accurate and equal inoculum size for all samples is also required (263, 267).

Sensititre Yeast One (TREK Diagnostic Systems)

Thermo Scientific™ Sensititre™ YeastOne™ YO9 AST Plate was originally developed using reference microdilution methods. So far, this method has been developed for susceptibility testing of nine antifungals for non-fastidious yeasts, including species of *Candida*,

Cryptococcus, and *Aspergillus* (274). The Sensititre™ YeastOne™ is an *in vitro* susceptibility test that needs an incubation temperature of 35°C for 24h for *Candida* isolates., 48-72h for *Aspergillus* ones, and 72h for *Cryptococcus* strains. The method is based on a colorimetric reaction. In the presence of cell growth, the colorimetric alamarBlue™ agent turns to purple color. The MIC is visually determined by observing a color change at the lowest antifungal concentrations that shows inhibition of growth. This method is easy to use and usually provides reliable results (275), but it needs at least an overnight incubation time (275). Up to now, several studies investigated the performance of the Sensititre™ YeastOne™ YO9 for AFST of yeasts. One of the first studies used 1,176 clinical isolates of yeasts and yeast-like organisms. The findings showed a high agreement of 92-100% between results obtained by Sensititre™ YeastOne™ YO9 (24h incubation time) and the CLSI method (48h incubation time) (275). However, for *Cryptococcus neoformans* isolates this method showed a lower agreement with results obtained by the CLSI method (76%), followed by *C. albicans* (87-90%) (275). Pfaller *et al.* (2004) designed another study to test the susceptibility of 300 *Candida* clinical isolates for fluconazole, voriconazole, posaconazole, and ravuconazole by Sensititre Yeast One and compared the results with those obtained by the CLSI method. They found a high agreement of 95.4% between both methods when the MIC was determined after 24 h with Sensititre Yeast One and after 48h with the CLSI method (274).

MERLIN *MICRONAUT*™ system

MERLIN *MICRONAUT*™ system (Bruker Daltonics GmbH, Bremen, Germany) is a microplate system developed for the susceptibility testing based on the growth assay of microbial in the presence of antibiotics. *MICRONAUT* system is now available for *in vitro* AST/AFST for the wide range of clinically important microorganisms against more than 200

antibiotics and common antifungals in the European diagnostic laboratories. This method requires less hands-on-work, but it is culture-dependent, and based on test microorganisms, it needs 24- 48h incubation time at 30-37 °C. The susceptibility of the microorganisms is interpreted by MICRONAUT software (https://www.merlin-diagnostika.de/en/index.html).

The Vitek 2 System (BioMérieux)

The Vitek 2 system is a fully automated system that is widely used in clinical laboratories for identification and susceptibility tests. This system uses cards containing serial twofold dilutions of different antifungals, i.e. amphotericin B, fluconazole, flucytosine, voriconazole, anidulafungin, and micafungin. Several studies showed that the Vitek 2 system provides reliable results for antifungal susceptibility testing of yeast species. For instance, in 2010, Cuenca-Estrella *et al.* tested 154 clinical yeast isolates of *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *Clavispora lusitaniae* (= *C. lusitaniae*), *Cryptococcus neoformans*, *Debaryomyces hansenii* (= *C. famata*), *Dipodascus capitatus, Kluyveromyces marxianus* (= *C. kefyr*), *Meyerozyma guilliermondii* (= *C. guilliermondii*), *Pichia kudriavzevii* (= *C. krusei*), and *Rhodotorula mucilaginosa* by the Vitek 2 system and results were compared with the CLSI and EUCAST methods. The Vitek 2 MIC results were obtained after 14 and 18 h incubation time and an essential agreement of > 97% for *Candida* species and 92% for *Cryptococcus neoformans* was obtained (276). A similar experiment was set up to evaluate the Vitek 2 system for both identification and antifungal susceptibility testing of several yeasts species (277). In this study, 32 clinical isolates of *C. albicans*, *C. glabrata*, *C. haemulonii*, *C. parapsilosis*, *C. tropicalis*, *Cl. lusitaniae*, *K. marxianus, M. guilliermondii*, *P. anomala* (= *C. pelliculosa*), and *P. kudriavzevii* were used to determine MICs against amphotericin B, flucytosine, voriconazole, and fluconazole after an

average incubation time of 15h. The results were compared with those obtained by the CLSI and EUCAST methods and full agreement was obtained between Vitek 2 and these reference methods (277). Recently, 172 *Candida* isolates of *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, and *P. kudriavzevii* were identified and tested for AFST by different methods (278). In this study, the Vitek 2 system was used to analyze the susceptibility of these isolates against fluconazole and amphotericin B. Incubation times of 9 and 27 h was needed for the Vitek 2 system based on the rate of growth in the drug-free control well. An agreement of 94% and 99% was observed between Vitek 2 system and the microdilution methods for fluconazole and amphotericin B, respectively (278). Taken together, the Vitek 2 system is presently the fastest method used in the clinical laboratories for the AFST of *Candida* spp. using an average incubation time of 15h (277, 278).

Flow Cytometry for antifungal susceptibility testing

Several recent studies investigated the application of Flow Cytometry (FC) for rapid antifungal susceptibility testing. This approach is based on inhibition of the growth of the cells by antifungal drugs. The *Candida* cells are labeled with a fluorescent probe such as propidium iodide (PI) or FUN-1, and then exposed to different concentrations of antifungals. In this method, a short incubation time of 4-9 h is used and compared to results from the CLSI and EUCAST methods that take 24-48h. After 4-9 h of incubation, the number of cells is measured by FC and the results of (lack of) growth of the cells in the presence of serial antifungal dilutions are compared with the control without antifungals. This method is expensive as it needs fluorescent probes, is complicated to use and not validated yet to be applied in routine clinical laboratories (279, 280). In 2005, Pina-Vaz *et al.* used FC to test the susceptibility of 63 clinical isolates of *Candida* species and *Cryptococcus neoformans* against voriconazole, itraconazole, and caspofungin. The cells were labeled with two fluorescent probes, propidium

iodide (PI) and FUN-1. Labeled cells were exposed to antifungals at different incubation times of 1, 2, 4, and 6h. The results showed that FUN-1 appears to be an excellent fluorescent probe for susceptibility testing getting results between 1 and 6h. Additionally, they showed that FC is a fast and reliable alternative approach for the classical microdilution method (279). Another study evaluated FC for AFST of one reference strain and 10 clinical isolates of *Cryptococcus neoformans* and *Cryptococcus gattii* against amphotericin B (281). The cells were stained with propidium iodide (PI) and the MIC of 0.06 to 2 µg/ml measured by FC was similar to the findings obtained by the reference microdilution methods (281).

Table 3. Differences between conventional and automated microdilution methods used in routine clinical laboratories and/or research use only (RUO).

Parameters	CLSI method	EUCAST method	Etest	Sensititre YeastOne (SYO)	Vitek 2	MBT ASTRA
Range of applicability	Yeasts and molds	Yeasts and molds	Yeasts and molds	Yeasts and molds	Yeasts	Yeasts
Medium	RPMI 1640 medium: Supplemented with 0.165 Mol/L MOPS + 2% glucose	RPMI 1640 medium: Supplemented with 0.165 Mol/L MOPS	RPMI agar plate	Calorimetric indicator (alamar Blue) + REDOX indicator	NA	RPMI 1640 medium: Supplemented with 0.165 Mol/L MOPS
Inoculation size	0.5-2.5 × 10^5 cells/ml	0.5-2.5 × 10^5 cells/ml	Covered whole plate with a suspension of MCF= 0.5 of respective isolate	1.5-8.0x10^3 Cells/ml	NA	0.5-2.5 × 10^5 cells/ml
Incubation time	24- 48h	24- 48h	24- 48h	24- 48h	12- 24h	6h
Shape of microplate	Round bottom plate	Flat bottom plate	NA	NA	NA	NA
MIC interpretation	Visual	Spectrophotometry	Visual	Visual	Automated	Automated
Format	BMD	BMD	Agar- based	BMD	BMD (AST-YSO6 CARDS)	BMD
Trailing growth	Yes	Yes	Yes	Yes	No	Yes
Application	Routine clinical laboratories	Routine clinical laboratories	Routine clinical laboratories	Routine clinical laboratories	Routine clinical laboratories	RUO

NA: Not applicable; **MCF:** MacFarlane Cell; **BMD:** Broth microdilution method; **RUO:** Research use only.

In summary, several efforts have been made to develop rapid and accurate methods to detect resistant *Candida* species. However, a broadly applicable method that can detect resistant *Candida* spp. in less than a working day and that can be used in routine clinical laboratories has not been developed yet. Furthermore, such a novel method should also minimize hands-on-work and having the potential for automation. Hence, in this Ph.D. project, the MALDI Biotyper antibiotic susceptibility test rapid assay (MBT ASTRA) was optimized for testing against echinocandins of *Candida* species for the first time. This method is the most rapid available susceptibility testing approach that can detect resistant *Candida* isolates after a 6h incubation time. MBT ASTRA is now available for use in research laboratories and will be described in detail in Chapter 2.

Diagnosis

Identification of clinical yeast species is crucial to start rapid and proper treatment of patients. The clinical presentation of candidemia varies widely and ranges from asymptomatic to severe systematic infections. There are several challenges to detect and identify infections caused by *Candida* species, such as:

a) The sensitivity and specificity of the method used for identification is crucial. It needs to be possible to distinguish between different *Candida* species and detect even very few numbers of yeast cells in the samples.
b) The method should be convenient to apply and to be used by laboratory staff without complicated training.
c) Speed and costs involved, and the amount of hands-on work needed (282, 283).

The available laboratory methods are categorized into three main groups, namely culture-based methods, immunological methods based on antibodies and antigens analysis, and molecular methods that rely on DNA, RNA, and protein analysis.

Culture-based methods

For invasive candidiasis, blood culture (BC) is the gold standard method to detect and identify yeast cells in blood samples, but these are positive only in 40-50% of cases (284, 285). Depending on the *Candida* species more than one day may be needed for BCs to show a positive result. As time is crucial for the treatment of patients suffering from invasive candidiasis, increasing the sensitivity of blood culture and shortening of incubation time are obtained by using fungal selective media (1, 284–286). Accordingly, Ericson and collaborators (2012) analyzed the performance of three different blood culture vials; Bactec Mycosis IC/F, BacT/Alert FA, and BacT/Alert FN. Their results showed the high sensitivity of BacT/Alert FA vials that were able to detect the highest number of positive samples (285). In contrast, Bactec Mycosis IC/F had a better performance when blood samples were concomitantly infected with both yeasts and bacteria. In conclusion, both Bactec Mycosis IC/F and BacT/Alert FA may improve the detection of *Candida* species from blood samples (285).

The germ tube test is another rapid assay to identify *C. albicans* and *C. dubliniensis* from other yeasts within 2-4 hours. This method is based on the growth of isolates of these two species and its ability to produce a germ tube in proteinaceous medium. Cells of *C. albicans* and *C. dubliniensis* are dissolved in the medium that contains sheep or human serum and incubated at 37 °C for 2-4 h. The process of generating a germ tube is based on the increased synthesis of proteins and ribonucleic acid resulting in the development of a short hyphal outgrowth from the yeast cells. Since almost 5% of *C. albicans* and *C. dubliniensis* isolates do not make a germ tube, the sensitivity of this method does not reach 100%. Additionally, *C. tropicalis* may be

wrongly identified as *C. albicans* or *C. dubliniensis* due to the formation of pseudohyphae that might be interpreted as germ tubes (287).

Immunological-based methods

Nonculture-based diagnostic methods are based on *Candida* antigens/antibodies assays, and serological markers (i.e. mannan, antimannan and (1,3)-β-d-glucan) (1, 288–290). Two antigen-based tests are in common use. The first one is called Platelia *Candida* Ag test and is developed by Bio-Rad Laboratories. This method detects mannan, one of the main components of the *Candida* cell wall, in serum samples using the ELIZA format (291, 292). Sensitivity and specificity of this method are about 58% and 93%, respectively (291). The second method detects 1,3-β-D-glucan (BDG), the second most common *Candida* cell wall component, in serum samples with the ELIZA format. This assay has been developed by four manufacturers and the Fungitell test (Associates of Cape Cod Inc., East Falmouth, MA, USA) is most popular. Sensitivity and specificity of this method are 77% and 85%, respectively (292, 293). Both antigen-based tests are used to diagnose invasive candidiasis in hematological and ICU patients. The advantages of these methods are the time needed for identification which is faster than radiological detection and positive blood cultures that take days. The disadvantages of these methods are: a) they need to be repeated twice or three times per week due to short-lived antigens in blood after presenting antibodies, and b) false-positives may be detected in different conditions like hemodialysis, abdominal surgery, treatment with β-lactam antibiotics, and the concomitant presence of lipopolysaccharide due to the presence of Gram-negative bacteria (293).

Two antibody-based assays are in use, one is used to detect antibodies against mannan for the identification of invasive candidiasis (294), and the second one is exclusively used to detect *C. albicans* by detecting antibodies against the germ tube (CAGTA) (295). The anti-mannan

antibody test is an ELIZA-based assay developed by Bio-Rad Laboratories and shows sensitivity and specificity of 59% and 83%, respectively. The anti-germ tube test assay is an indirect immune fluorescence method developed by Virvell Laboratories, Granada, Spain, and has a wide range of sensitivity and specificity ranging between 77% to 89%, and 91% to 100%, respectively (291, 295). The advantage of both antibodies-based methods is their rapidness. However, a combination of both methods based on antibody and antigen detection may increase sensitivity and specificity of *Candida* identification (293).

Molecular based- methods

DNA-based methods have been developed either to identify invasive candidiasis in high-risk patients or to classify intraspecies variation and isolate clustering. These methods are more rapid and accurate than traditional phenotypic assays, and they have been recently used as gold standard methods in routine clinical laboratories (267, 287). Among the molecular methods, polymerase chain reaction (PCR) is commonly used for yeast species identification. A frequently used PCR technique is the standard PCR based on amplification and sequencing of a target locus which usually are highly conserved regions of rDNA. The eukaryotic rRNA is encoded by the 18S, 5.8S, and 28S rRNA genes that are transcribed as a unit by RNA polymerase I, and the internal transcribed spacer (ITS) regions, particularly ITS1 and ITS2 (296, 297). The ITS1 and ITS2 nucleic acid sequences show a high diversity between some *Candida* species and can be used for identification at the species level. The 18S nuclear ribosomal small subunit rRNA gene is commonly used for phylogenetic studies. The 28S nuclear ribosomal large subunit rRNA gene (LSU) is also used to distinguish between species, either alone or together with ITS (267, 291, 296–298).

Endpoint PCR-based amplification is commonly used to identify pathogenic agents from culture media or direct from patient samples, while due to its limitation to detect rare and/or

newly emerging yeasts, it is not widely used for identification of fungi in the clinic (299, 300). Other available PCR methods using for yeast identifications are nested and semi-nested PCR, PCR-enzyme immunoassay, real-time PCR, and multiplex PCR. Real-time PCR is a faster and more straightforward approach, but like other molecular techniques is not cost-effective (290). More recently, in a new study performed by Arastehfar and collaborators (2018), a novel multiplex endpoint PCR was evaluated to identify 172 *C. auris* stains and its close relatives, including *C. duobushaemulonii***,** *C. haemulonii*, and *C. pseudohaemulonii,* derived from mice blood and tissue. Additionally, 192 isolates of other clinically important *Candida* species, including *C. africana*, *C. albicans*, *C. glabrata*, *C. metapsilosis*, *C. orthopsilosis*, *C. parapsilosis*, *C. tropicalis*, and *Pichia kudriavzevii*, were analyzed. Their results revealed positive rates of this new PCR method for mice blood spiked with yeast cells and infected mice tissue of 28.6 and 92.9%, respectively (301).

Most available commercial PCR kits have been designed to identify a single species and are unable to identify rare yeast species that are emerging (302). Therefore, this drawback has resulted in a demand to develop pan-fungal kits (broad-spectrum PCR primers) that can detect concomitantly both common and less common species from patient specimens. However, to detect new emerging *Candida* species the available pan-fungal kits require to be updated. Other disadvantages of the pan-fungal PCR assays are the interpretation of results which requests professional training and a possibility of detection of non-yeast pathogens due to the high sensitivity of this method (267, 303).

Fragment Length Polymorphism (AFLP) is another molecular-based method that is not widely used for yeast identification, but rather it is used to evaluate intraspecies variation and isolate clustering, e.g. in outbreaks. This method is rapid and accurate with a relative high reproducibility. The AFLP method has a wide range of applications and no sequence information is needed for primer construction. However, it requires purified, high molecular

weight DNA and bands with different intensities may be generated that must be standardized for acceptance in the analysis (146, 267, 304).

Overall, molecular diagnostic methods are more rapid and accurate than conventional biochemical methods in patients suspected of having invasive candidiasis. Usually, these methods have a high sensitivity and specificity to identify invasive candidiasis agents in high-risk patients. However, they are still considered as expensive with an intensive workload for laboratory personnel. Furthermore, not all PCR-based methods can be used directly on human blood samples, because DNA of human blood cells, and if there is contamination, DNA of other microorganisms, are co-extracted with the DNA of yeasts from blood samples and this may cause cross-reacting and problems in the identification of the yeast species. Therefore, due to this difficulty more precise and larger studies are needed to develop accurate and specific primers (305). Some available DNA extraction kits have solved this drawback, but they still need several steps for sample preparation which is time-consuming (267, 306). Furthermore, the value of these methods compared to other available diagnostic approaches needs to be addressed.

Other methods, like T2 magnetic resonance (T2MR) (T2 Biosystems, Lexington, the USA), have been invented for microbial identification. T2MR is a newly developed qualitative molecular method combining magnetic resonance with nanotechnology methods and is used to diagnose *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *P. kudriavzevii* in patients suffering from invasive candidiasis (307, 308). T2MR is FDA-approved to diagnose *Candida* species in whole cultured blood samples in approximately 5h with a sensitivity and specificity of 91.1% and 98.1%, respectively (307). Besides the short time of identification, it has been investigated to monitor candidemia clearance after antifungal treatment in a clinical trial (307, 309). Due to the presence of a few yeast cells (even one cell per ml) in infected blood samples,

monitoring of candidemia by T2MR is difficult by using blood cultures. Finally, this method is very expensive, which is considered as a main drawback (307, 309).

Fourier-transform infrared spectroscopy (FTIR)

FTIR spectroscopy is based on the analysis of an acquired infrared spectrum from a sample in a solid, liquid, and the gas form. This approach is used for identification of microbes based on collecting spectra with high resolution over a wide spectral range (348–4000 cm^{-1}) (310, 311). Absorbed IR radiation is characterized based on the wavelength and is used to identify molecular components and structures (311). FTIR spectroscopy is a rapid, reliable, cost-effective, and time-efficient method that is mostly used for the identification of different microorganisms (311, 312), but it is one of the less used methods in strain typing. It was firstly applied for the identification of *S. cerevisiae* isolates and differentiation between *S. cerevisiae* and non-*S. cerevisiae* strains (313). Thereafter, the application of FTIR spectroscopy was evaluated for bacterial strain typing (314). So far, FTIR spectroscopy has not been widely used for the typing of *Candida* species. One study used FTIR spectroscopy as a phenotypic method to type *C. albicans* derived from patients in intensive-care units (315). They acquired 79 *C. albicans* strains within four months from nine patients hospitalized in two intensive care units, and FTIR could accurately cluster the isolates derived from each patient in a separate group (315).

A new version of FTIR spectroscopy, the IR Biotyper (Bruker Daltonics GmbH), has been recently developed for microbial strain typing (316). The IR Biotyper has been innovated for the detection of different microbial outbreaks to implement proper hygiene measures in hospitals. This method functions by the analysis of fingerprints of molecular vibrations in the infrared spectrum. Whole-cell lysis is performed on biomass obtained from overnight cultured isolates. The spectrum is generated based on the differences of surface cell polysaccharides,

and the individual spectrum is used for typing of microbes. This method is culture dependent and the results are released between one and three hours (317). Since molecular methods are expensive and time-consuming, IR Biotyper has a number of advantages and might become a useful tool for isolate typing to be used in hygiene control in clinical laboratories in the future (318).

A few studies focused on the application of the IR Biotyper in strain typing. For instance, Dinkelacker *et al*. (2018) used this method for typing 68 clinical *Klebsiella* isolates. Isolates were analyzed by whole genome sequencing (WGS), MALDI-TOF MS, and the IR Biotyper. The last method showed good performance compared to the WGS reference method and correctly recognized the clonal relationship of the isolates. The authors recommended that this new method is a valuable tool for the detection of transmission routes in hospitals to improve hygiene control. MALDI-TOF MS seems less useful for strain typing. In this study, WGS-based phylogenetic analysis showed that the *Klebsiella* isolates belonged to three species, namely *K. pneumoniae*, *K. variicola*, and *K. quasipneumoniae*. MALDI-TOF MS was used to identify these three species based on the shifts of certain mass peaks. Peak shifts indicated the differences of amino acids between *K. pneumoniae* and *K. variicola*. Further analysis showed that the peaks belonged to three ribosomal proteins (S15p, L28p, L31p) and one stress response protein (YjbJ) (316). More recently, Martak *et al*. (2019) evaluated the application of the IR Biotyper to assess the clonal relationship of Gram-negative bacilli in a hospital outbreak. They tested several isolates of *Pseudomonas aeruginosa* (n = 100), *Klebsiella pneumoniae* (n = 16), and *Enterobacter cloacae* (n = 23), and *Acinetobacter baumannii* (n = 20) by the IR Biotyper and the results were compared with Multi-Locus Sequence Typing analysis (MLST) as the reference method. The IR Biotyper could correctly detect the spread of clones of *P. aeruginosa*, *K. pneumoniae*, *E. cloacae*, but was found to be less useful for *A. baumannii* typing due to misclassification of three out of 20 isolates. Overall, they reported that this new

method may be helpful in the rapid detection and analysis of microbial outbreaks in hospital settings (318).

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS)

Mass Spectrometry is a semi-quantitative method developed for the identification of different microorganisms (319). Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been recently revolutionized and is currently widely employed in routine clinical laboratories (319). Over the last two decades, due to its rapidity and accuracy, this method has turned into one of the most widely used diagnostic methods in clinical laboratories in Western countries (319–321). There are two companies that developed MALDI-TOF MS, namely MALDI Biotyper (Bruker Daltonics, Bremen, Germany), and VITEK MS (BioMérieux, Marcy l'Etoile, France). Each company has built up its own spectral database (321). MALDI Biotyper is used to identify yeasts, filamentous fungi and bacteria frown at agar plates or broth blood cultures based on the analysis of ribosomal proteins (319, 322). MALDI Biotyper is a cultured-based method and was explored in this thesis for the identification and antifungal susceptibility testing of three *Candida* species, namely *C. albicans*, *C. glabrata*, and *C. auris* against echinocandins (Chapters 2, 3, 4, 5).

There are three different protocols of extraction of macromolecules to identify microorganisms by MALDI-TOF MS:

a) **Direct Identification (DT):** In this method the biomass of the cultured microorganism from agar plates are directly spotted onto a polished steel target plate (Bruker Daltonics) and overlaid with 1 µl MALDI matrix (10 mg/ml of α-cyano-4-hydroxy-cinnamic acid [α-HCCA] in 50% acetonitrile–2.5% trifluoroacetic acid; Bruker Daltonik). This method is usually used for Gram-positive bacteria that have a thin cell wall (322, 323).

b) **Extended Direct Identification (eDT):** This extraction method is similar to the DT method, but requires one additional step, namely the addition of 1 µl formic acid 70% to the biomass before overlaying it with the MALDI matrix. This method is often used for Gram-negative bacteria and yeasts that have a thicker cell wall (322, 323).

c) **Ethanol (EtOH)-formic acid (FA) extraction (ET):** The full extraction method can be used for all microorganisms to obtain the most accurate results. This method is recommended for fungi (yeasts and filamentous fungi) and Gram-negative bacteria (320). In this workflow, a colony of the cultured microorganisms is added into an Eppendorf tube and then a volume of 1200 µl ethanol 70% is added and the suspension is centrifuged. Next, the supernatant is discarded and according to the size of the pellet, an equal amount of formic acid 70% and acetonitrile 100% is added. After centrifugation, 1 µl of supernatant is pipetted on the target plate. The target is dried at room temperature and overlaid with the matrix. The prepared target is introduced to the MALDI-TOF MS machine (320, 323).

To diagnose clinical isolates derived directly from broth blood cultures, several methods have been developed. Among the available published methods, the MALDI Sepsityper kit (Bruker Daltonik GmbH, Germany) has shown a good performance for the identification of microorganisms (160). The full protein extraction procedure is performed on samples and 1 µl of supernatant is placed on the target and covered by matrix (160, 324). Matrix is an essential component to use converting macro molecules, such as proteins and lipids, into the ions to be analyzed by MALDI-TOF MS. Several matrixes have been developed for use in MALDI-TOF MS. The most common one used for peptides, lipids, and nucleotides is HCCA (10 mg/ml of α-cyano-4-hydroxy-cinnamic acid [α-HCCA] in 50% acetonitrile–2.5% trifluoroacetic acid) (325–328), followed by sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) that is used for peptides and protein identification (329–331). The third common matrix is DHB (2,5-dihydroxybenzoic acid); it is widely used for peptides, nucleotides, oligonucleotides, and

oligosaccharides (328, 332, 333). Additionally, a couple of modified or combination of matrixes has been tested in order to improve the identification of different microorganisms with variant molecular weights by MALDI TOF MS (334–337).

During the analysis by MALDI-TOF MS, sample protein molecules vaporize due to the bombardment of laser energy and they turn into small ionized positive molecules. These ions are separated based on their mass-to-charge ratio and detected by a linear detector. The created spectra are compared with those available in the reference library database to identify yeasts (and/or other microorganisms) at the species level (Figure 3) (266, 338). MALDI-TOF MS spectra acquired with a Microflex LT/SH mass spectrometer (Bruker Daltonik) are calibrated with the bacterial test standard (BTS; Bruker Daltonik) in a mass range between 2,000 and 20,000 Da (266).

MALDI-TOF MS shows superior performance over other diagnostic methods. For instance, the method is fast and able to identify many different microorganisms in less than one hour with the complete extraction method. The accuracy of this method is high with a sensitivity of 91 to 100% and a specificity of 100% (339). Lack of identification and misidentification usually results from either insufficient database entries or inappropriate sample preparation (339). The identification of isolates with a complex cell wall composition is usually sub-optimal and needs a full extraction. As an example, we can refer to *Mycobacterium* species, and some Gram-positive bacteria, like *Streptococcus pneumoniae*, members of the *Streptococcus oralis/mitis* group, and *Listeria* species, that need high quality extraction to be detectable by MALDI-TOF MS. Furthermore, the identification of anaerobic bacteria has been challenging (340–343). The identification of fungi has been problematic due to the presence of their thick cell wall. Therefore, the full extraction method is recommended to be used for most fungi (339). So far, several studies have been performed to evaluate the MALDI-TOF MS method for the identification of different *Candida* species as well as filamentous fungi.

Presently, the majority of clinical fungal isolates can be properly detected on both agar plates and broth blood culture (160, 320, 344, 345).

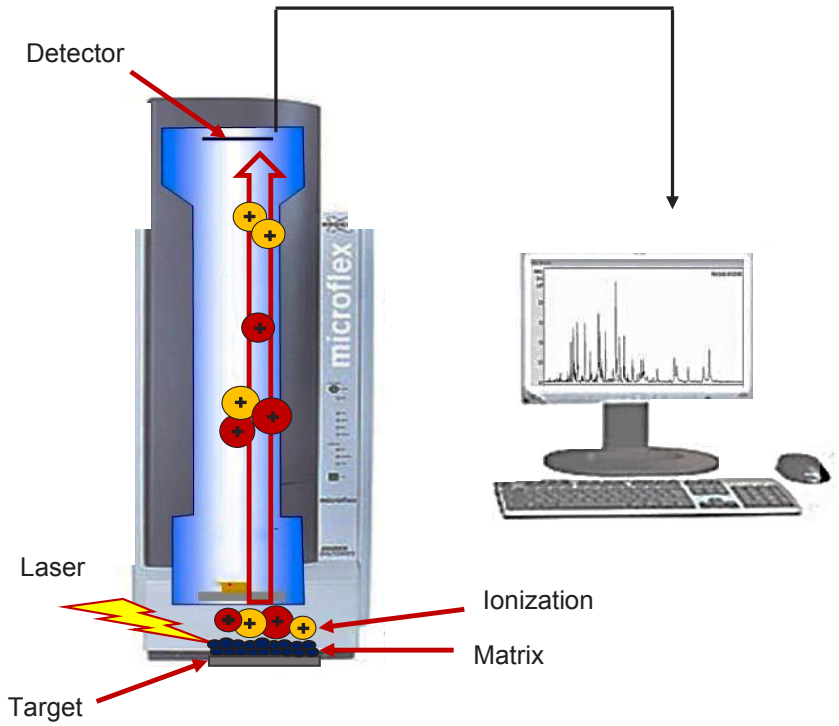


Figure 3. A schematic function of the MALDI-TOF MS. This figure is taken from Patel R (2015) [338].

Marklein *et al.* (2009) performed one of the initial experiments using MALDI-TOF MS to identify 267 yeast isolates belonging to genera like *Candida*, *Cryptococcus*, *Geotrichum*, *Pichia Saccharomyces*, and *Trichosporon*. They reported that 247 out of 267 isolates were correctly identified, resulting in a sensitivity of 92.5%. The remaining 20 isolates could not be identified as they were not included in the database (282). Taj-Aldeen and collaborators (2014) tested the performance of MALDI-TOF MS on 201 clinical *Candida* isolates from a Qatar-

based hospital. They reported that all *Candida* isolates were correctly identified with a high log score value of ≥ 2 (99.5 %), excluding one *C. dubliniensis* which was detected with a log score of 1.7 < value < 2.0 (346). Later, another study was performed by Galan *et al.* (2015) on 600 clinical isolates belonging to 9 genera, including 43 *Candida* species. MALDI-TOF MS identified 580 (96.7%) of the isolates correctly. The results obtained by MALDI-TOF MS were compared with those obtained by sequencing of the ITS regions of ribosomal DNA and assimilation of carbon compounds by ID 32C cards. An agreement of 100% was obtained between MALDI-TOF MS and carbon compounds (ID 32C) for the clinically most common *Candida* species, like *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *P. kudriavzevii*. A high concordance was observed between MALDI-TOF MS and molecular methods for the identification of clinical yeast isolates (347). In general, a large number of studies focused on the identification of different clinical yeast isolates by MALDI-TOF MS, and the results indicated a high sensitivity of this method that is able to diagnose different *Candida* spp., including *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. metapsilosis*, *C. nivariensis*, *C. orthopsilosis*, *C. parapsilosis*, *C. tropicalis, P. kudriavzevii*, and *Kluyveromyces marxianus* (346, 348, 349). The *Cryptococcus neoformans* species complex previously included only two sibling species, *Cryptococcus neoformans* and *Cryptococcus gattii*. These species are divided into seven major molecular types or, more recently, seven species (304, 350). The species differ in the diseases they cause, their virulence, response to antifungals and epidemiology. MALDI-TOF MS was found to be a rapid method for the identification of members of the *C. neoformans* and *C. gattii* species complex. A study performed by Firacative *et al.* (2012) correctly identified all of 164 *C. neoformans* and *C. gattii* isolates by MALDI-TOF MS (351). Another study used 82 *Cryptococcus* isolates, including 72 *C. neoformans* and 10 *C. gattii*. Eighty-one out of the 82 *Cryptococcus* isolates were correctly identified by MALDI-TOF MS (352). Hagen and collaborators (2015) recognized two new species in the

Cryptococcus neoformans species complex, namely *C. neoformans* (= *C. neoformans* var. *grubii*) and *C. deneoformans* (= *C. neoformans* var. *neoformans*). They tested nine recently identified species of the *C. neoformans*/ *C. gattii* complex by MALDI-TOF MS. The results indicated that MALDI-TOF MS could successfully identify all nine species of the complexes, as well as a number of inter species hybrids (304). Aminnejad *et al.* (2016) performed a study to evaluate the capability of MALDI-TOF MS to also distinguish hybrid isolates within the *C. neoformans* and *C. gattii* species complexes. All hybrids belonging to different sero/mating types patterns were correctly identified by MALDI-TOF MS (353).

Another advantage of MALDI-TOF MS is the diagnosis of rare and new emerging yeast species. This, however, requires an updated database that contains the spectra of newly emerging *Candida* species. For instance, MALDI-TOF MS is now able to correctly identify *C. auris* from related species, like *C. haemulonii* (154, 354). Accordingly, one of the studies performed in this thesis was the identification of *C. auris* derived from SDA and broth blood culture using the MALDI Sepsityper kit (Bruker Daltonik) by MALDI-TOF MS according to the manufacturer's recommendations (Chapter 5) (160, 266, 355). Furthermore, the proficiency of MALDI-TOF MS in identification is not only limited to clinically important yeasts, but it has been also expanded to environmental yeasts and food-related species (356–359). MALDI-TOF MS is mainly used in Western countries due to the high cost to purchase an apparatus and its database. Moreover, MALDI-TOF MS has not yet been optimized to identify microorganisms directly from blood samples without culture, and also the accurate identification of two or more different microorganisms present in the same sample poses still a problem (290, 360).

Besides the use of MALDI-TOF MS to identify microorganisms, it has been recently considered as a novel and rapid method for antimicrobial susceptibility testing. The MALDI Biotyper antibiotic susceptibility test rapid assay (MBT ASTRA) has been developed lately to

detect bacteria resistant to different antibiotics in less than five hours (319, 361–363). For the first time, MBT ASTRA has been optimized for the detection of *C. albicans*, *C. glabrata* and *C. auris* that are resistant against echinocandins within 6 hours (Chapters 2, 3, 4, 5).

Thesis outline

The most important achievement of this thesis is the optimization of MALDI Biotyper antibiotic susceptibility test rapid assay (MBT ASTRA) for *Candida* species against echinocandins in six hours. This method and the process of optimization is described in chapter 2. MBT ASTRA is the most available rapid method for antibiotic/antifungal susceptibility testing (AST/AFST) and it is now ready to use in research. This method is potential to be automated and it will be introduced to routine clinical laboratories after further optimization and automization. Application of this method has been evaluated against echinocandins for clinically important *Candida* species, namely *C. albicans*, *C. auris*, and *C. glabrata* derived from agar plate, spiked blood samples, and then directly on the patient's blood samples. Three published articles relevant to MBT ASTRA are presented in separate chapters.

Identification of *C. auris* derived from spiked blood samples using the MALDI-TOF MS and up-to-date FDA approved database developed by Bruker Daltonic Company, Bremen, Germany is also explained in this thesis. In addition, MALDI-TOF MS is also able to detect hLF(1-11) peptide. Therefore, chapter 7 describes how MALDI-TOF MS is used to identify few numbers of *Candida* cells (1000 cells/ml) enriched with hLF(1-11) peptide directly from patient`s blood samples. This evaluation is a promising approach to obtain the most crucial goal in the clinical laboratory which is to detect *Candida* cells (1-10 cells/ml) directly from patient`s blood samples.

MALDI-TOF MS is used to type *C. auris* isolates belonging to four different clades compared to the IR Biotyper method as one of the biochemical typing methods. IR Biotyper (Bruker Daltonics GmbH) has been recently innovated to identify and detect microbial outbreaks in hospitals. So far, the IR Biotyper has not been used for the analysis of human pathogenic *Candida* yeasts. Since in less than one decade, several *C. auris* outbreaks have been reported in various hospitals worldwide, we explored for the first time the applicability of the IR Biotyper for strain typing of *C. auris* isolates and the results were compared with data obtained using other methods, like microsatellite assay, AFLP, ITS sequencing, and MALDI-TOF MS. The results are summarized in Chapter 6. MALDI-TOF MS has a strong power in the identification and AST/AFST of *Candida* species, however, it is less accurate for typing *Candida* isolates. The thesis ends with a discussion chapter (Chapter 8) and a summary (Chapter 9).

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Chapter 2

Development of MALDI Biotyper antibiotic/antifungal susceptibility testing rapid assay (MBT ASTRA) for *Candida* species against echinocandins

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Introduction

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is an easy, fast, affordable and high throughput technology that is widely used for the identification of microorganisms in routine clinical laboratories (1). The rapidness and accuracy of this method and the broad application in Western countries have stimulated its application in other fields. One example of such a new application is to use MALDI-TOF MS for antibiotic/antifungal susceptibility testing (AST/AFST) (2-4). Since reference microdilution methods take 24-48h for AFST of yeast isolates, the application of MALDI-TOF MS for AFST may reduce this time. Hence, a great deal of research focus on AST/AFST using MALDI-TOF MS.

In 2009, Marinach *et al.* were the first to set up a method to test the susceptibility of *Candida albicans* isolates to fluconazole by MALDI-TOF MS (5). They measured the proteins of *C. albicans* cells (8 susceptible, 8 resistant strains) when grown with and without different antifungal concentrations. The presence or absence of peaks using the full extraction method, and/or shifting of peaks in a mass range from 2,000 to 20,000 Da were analyzed after 15h incubation time. The approach was found to be rapid and accurate with 100% agreement with the Clinical and Laboratory Standards Institute (CLSI) method (5). However, this study only used a small sample size and only one *Candida* species. Additionally, the 15h incubation time, plus preparation and analysis times did not result in a shortening of the time needed for the analysis. Accordingly, in another study, a rapid AFST MALDI-TOF MS-based approach compared spectra profiles acquired in the presence of twofold serial dilutions of caspofungin and in a control without the antifungal. The authors analyzed wild-type and *FKS* mutant isolates of *Candida* species, including *C. albicans*, *Candida glabrata*, *Candida parapsilosis*, and *Pichia kudriavzevii* (= *C. krusei*) and of *Aspergillus* species, including *Aspergillus fumigatus* and *Aspergillus flavus*. Thirty-four *Candida* and 10 *Aspergillus* isolates were exposed to caspofungin for 15h. A composite correlation index (CCI) matrix was calculated from the spectral profiles and compared to results obtained with the CLSI method. In this method, CCI values about one and zero showed high agreement and diversity of the spectra, respectively. The acquired spectra were compared to each of the two extreme concentrations of caspofungin (zero or 64 µg/ml) and used to determine the minimal profile change concentration (MPCC) of this drug. The MPCC value refers to the spectrum obtained at the lowest drug concentration that indicated the highest similarity to the spectrum acquired at the highest concentration of caspofungin compared to the spectrum acquired at the zero caspofungin concentration. The essential agreement between the MPCC as observed by MALDI-TOF MS and the minimal inhibitory concentrations (MIC) values as obtained by the CLSI method was 100% for both *Candida* and *Aspergillus* species. A categorical agreement of 94.1% was acquired between the methods; only two susceptible *P. kudriavzevii* were misclassified as intermediate by MALDI-TOF MS (6). In 2013, the same group developed a simplified version of their innovative rapid AFST by MALDI-TOF MS (7). Here, they evaluated the profile changes in the presence or absence of three different caspofungin concentrations [0 (null), 0.03 (intermediate), and 32 (maximal) µg/ml] after 3h incubation. They tested 65 *C. albicans* isolates (52 susceptible, 13 resistant) by calculating individual composite correlation index (CCI) matrices. In general, a high agreement of 98.4% was obtained between this rapid AFST approach and the CLSI method (7). In 2017, this group applied their innovative rapid AFST using MALDI-TOF MS to test AFST of *C. glabrata* against anidulafungin and fluconazole. Serial dilutions were made of the respective antifungals and the results were released after a 3h incubation time. The outcomes were compared with the CLSI method and *FKS1* genotype assay. The high reproducibility of this method was confirmed for fluconazole with an agreement of 96.2%, but a lower agreement of 85.0% was obtained for anidulafungin (8).

The MBT-RESIST (Resistance Test with Stable Isotope-Labeled Amino Acids) was a new rapid AST method developed in 2013 by Bruker Daltonik GmbH (Bremen, Germany) (9). They used stable isotope labeling against *Staphylococcus aureus* to detect resistant isolates. Three different concentrations of proteinogenic amino acids, including antibiotics, were used with a final concentration of 3.5-10^6 bacteria per ml. Incubation was done for 3h. The changes in the profile spectra and the presence or absence of peaks in the mass range between 2,000 and 20,000 Da were analyzed by MALDI-TOF MS. Resistant isolates could grow in the presence of the antibiotic and the incorporation of the labeled amino acids increased the protein masses that were detected by MALDI-TOF MS. However, this approach was not yet tested for yeasts and due to the requirements of specialized media, it was not optimized for other bacteria, and, therefore, the method was not introduced to the clinical laboratories (9). Later, in 2014, a novel approach was developed by the same company called the MALDI Biotyper antibiotic/antifungal susceptibility test rapid assay (MBT ASTRA), which will be described in detail below.

MALDI Biotyper antibiotic/antifungal susceptibility test rapid assay (MBT ASTRA)

MBT ASTRA is a novel rapid method to detect antibiotic/antifungal susceptibility in a few hours by MALDI-TOF MS. This approach is a semi-quantitative assay based on the growth of the cells in the control compared to that in the presence of twofold serial dilutions of the antibiotic/antifungal to be tested after a few hours of incubation. The cells are collected and extracted as described elsewhere (10-12). One microliter of the cell lysates is spotted 2 or 4 times onto a polished steel MALDI-target plate and covered by 1 µl MALDI-matrix (10 mg/ml of α-cyano-4-hydroxy-cinnamic acid [α-HCCA] in 50% acetonitrile–2.5% trifluoroacetic acid; Bruker Daltonik) including a standard. This standard is used for further analysis by MS ASTRA prototype software (proprietary of Bruker Daltonik), and differs according to the nature of

microorganisms (bacteria or yeasts) to be tested. The MALDI-TOF MS measurement is performed in a mass range between 2,000 and 20,000 Da (10, 12, 13) and the acquired spectra are analyzed by MS ASTRA prototype software. This prototype has been written in R according to the procedure described by Lange *et al.* (2014) to analyze spectra based on the AUC (area under the curve) for each incubation setup (14, 15). The AUC for an individual concentration of used antibiotic/antifungal is normalized to the standard and compared with the control. The AUC of the antibiotic/antifungal setup when compared to that of the control is used to calculate the relative growth (RG) of the isolate as follows: $RG = (AUC_{RPMI+caspofungin})/(AUC_{RPMI})$ (10, 11, 16). The RG threshold is used to determine the unique cutoff value for each species against the respective antibiotic/antifungal. The interpretation of the results is based on the RG threshold and the cutoff value. Isolates that grow above the defined cutoff threshold are considered as resistant and the isolates showing an RG below this cutoff threshold are classified as susceptible (2, 10, 17).

MBT ASTRA was firstly used to detect resistance of *Klebsiella* species against meropenem (2). A serial dilution of meropenem ranging from 0.025 to 32 µg/ml was prepared. Cells were inoculated in 0.5 McFarland standard density and incubated for 1, 2 and 3h on both brain-heart infusion (BHI) medium (Heipha, Germany) and blood culture bottles (BD BACTEC Plus Aerobic/F; Becton Dickinson, Germany). The results were compared to the MIC obtained by the EUCAST method (2). At a meropenem concentration of 8 µg/ml, a sensitivity and specificity of 97.3% and 93.5% were calculated after 1h of incubation, respectively (2). In a follow-up study, Jung *et al.* (2016) evaluated the performance of MBT ASTRA *Enterobacteriaceae* isolates (30 isolates from their collection and 99 patient-derived blood cultures) against different antibiotics, such as gentamicin, ciprofloxacin, cefotaxime, and piperacillin-tazobactam. Excluding misclassification of one resistant isolate against ciprofloxacin and five isolates against piperacillin-tazobactam, the rest of the resistant isolates

were correctly detected using broth blood cultures after 4 hours of growth (4). Subsequently, Sparbier *et al.* (2016) showed the performance of MBT ASTRA for different antibiotic-species combinations, such as cefotaxime-*E. coli*, meropenem-*K. pneumoniae*, meropenem-*P. aeruginosa*, tobramycin-*K. pneumoniae*, tobramycin-*P. aeruginosa*, and tobramycin-*A. baumannii*. Isolates were tested by MBT ASTRA derived Columbia blood agar plates (BD Diagnostics Systems, Germany). The results from the susceptibility tests were released in a few hours for the various drug/microbe combinations. The tests for piperacillin-*E. coli* and ceftazidime-*P.aeruginosa* did not show promising outcomes and need further optimization (18). Overall, outstanding results were obtained and showed the hight applicability of the MBT ASTRA method to detect various bacteria species that were resistant to different antibiotics.

To determine the applicability of MBT ASTRA for Gram-positive bacteria, a first study was performed by Maxon *et al.* (2017) (19). They tested 35 *Staphylococcus aureus* isolates derived from positive blood cultures against ciprofloxacin, oxacillin, cefepime and vancomycin. Within 3h, they could successfully detect the majority of resistant isolates with an agreement of 95% compared to Etest (19). Thus, MBT ASTRA is potentially a very useful method for susceptibility testing of both Gram-positive and Gram-negative bacteria.

Follow-up experiments showed the usefulness of MBT ASTRA to perform susceptibility tests for *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species (20). As a next step, the applicability of MBT ASTRA was expanded to *Mycobacterium* species. In 2017, Ceyssens *et al.* analyzed the susceptibility of 39 *Mycobacterium tuberculosis* clinical isolates for rifampin, isoniazid, linezolid, and ethambutol, and 33 nontuberculous mycobacterial (NTM) clinical isolates against rifampin and ethambutol by MBT ASTRA (20). According to the number of antibiotics, every isolate was tested either fur times (*M.* tuberculosis, N = 156) or 2× (NTM, N = 66). The results were compared with those obtained by using the BACTEC MGIT 960 system

for *M. tuberculosis* and the Sensititre RAPMYCO and SLOMYCO panels for NTM. At an average of 6 days of incubation, all *M. tuberculosis* (156/156) and 98% of NTM (65/66) isolates were correctly characterized as either susceptible or resistant. Although MBT ASTRA could not significantly reduce the incubation time for *M. tuberculosis*, this method was able to detect resistant NTM isolates one week faster than other available routine AST methods used for these bacteria (20).

Further experiments, using a broader range of prokaryotic microorganisms, will throw more light on the applicability and accuracy of MBT ASTRA to further increase the use of this method into routine clinical laboratories. MBT ASTRA is a novel rapid method developed to detect resistant isolates in a short incubation time when compared to conventional microdilution methods.

Development of an MBT ASTRA protocol for yeasts: Despite the promising applicability of MBT ASTRA to detect susceptible and resistant bacteria isolates, there was no effort to evaluate the performance of this approach for *Candida* species in the past. Therefore, this Ph.D. project was designed to optimize MBT ASTRA for clinically important *Candida* spp. for the first time. The main aim of this project was to optimize MBT ASTRA as an approach that can reveal the results on AFST of yeasts in less than one working day in clinical laboratories. MBT ASTRA may significantly improve patient management due to shortened time to result of AFST of *Candida* isolates for various antifungals. This helps to select proper antifungal therapy and maximize survival of the patients (Figure 1) (21, 22). Ideally, MBT ASTRA should be rapid and user-friendly with minimal hands-on-work. In this thesis, three *Candida* species, namely *C. auris, C. albicans*, and *C. glabrata* were selected based on their clinical importance causing a broad range of infections, and MBT ASTRA was set up to perform AFST of echinocandins for these species.

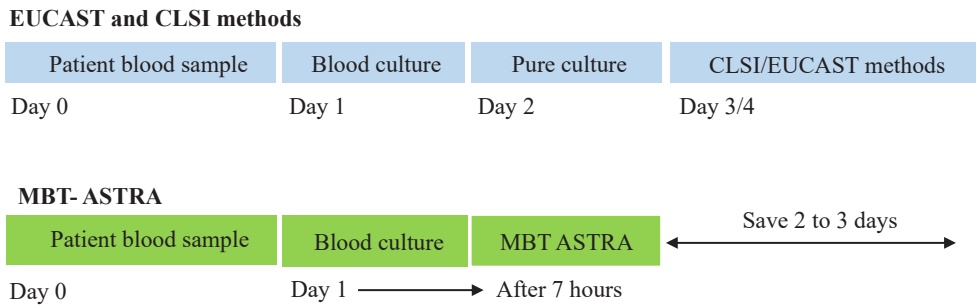


Figure 1. Comparison of time-to-result of MBT ASTRA with standard microdilution methods in routine clinical laboratories.

MBT ASTRA was set up according to the EUCAST method with a few modifications (23). The Roswell Park Memorial Institute Medium (RPMI 1640) with L-glutamine, without glucose and sodium bicarbonate (Sigma-Aldrich, Germany) supplemented with a final concentration of 0.165 Mol/L morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich, Germany) and 2% glucose (Sigma-Aldrich) was made fresh every week. The pH of the medium was adjusted to 7.0 using 1M sodium hydroxide at 25°C. Isolates were stored at -80°C and they were cultured on Sabouraud Dextrose Agar (SDA) for 24h at 35°C before performing the AFST experiment. Cell suspensions of McFarland 0.5 (Grant-bio-DEN-1, England) were prepared with yeasts cultivated on SDA. Based on the EUCAST recommendations, cell suspensions were further diluted 1:20 to get a final concentration between 0.5 x 10^5 and 2.5 x 10^5 CFU/ml (Table 3-chapter 1). Contrary to the EUCAST rules, the whole set up used a larger volume of 600 µl in the 1.5 ml Eppendorf tube. Additionally, a control, including RPMI 1640 medium and cell inoculum was made for every isolate. Incubation was done at 37 °C with shaking at 300 rpm in a ThermoMixer (Eppendorf, Germany) for six hours for all tested *Candida* species. This

study focused on echinocandins, i.e. caspofungin, anidulafungin, and micafungin, and depending on the *Candida* species different twofold serial dilutions of the used antifungals were prepared (Table 1).

Table 1. Breakpoints determined for *Candida auris, C. albicans*, and *C. glabrata* against caspofungin, anidulafungin and micafungin from Sabouraud Dextrose Agar (SDA) plates and broth blood culture by MBT ASTRA. Dark grey boxes show that there is no study yet regarding these species against respective antifungals on SDA or broth blood culture. Susceptible is shown by S, and resistant is shown by R.

		C. albicans **(On SDA)**	***C. glabrata*** **(On SDA)**	***C. glabrata*** **(On Broth Blood Culture)**	***C. auris*** **(On SDA)**	***C. auris*** **(On Broth Blood Culture)**
Caspofungin	Twofolds Serial Dilutions	0.125- 4 µg/ml	0.125- 4 µg/ml		0.125- 4 µg/ml	0.125- 4 µg/ml
	Recommended Cutoff Value	S ≤ 1 R > 1	S ≤ 0.5 R ≥ 1		S < 2 R ≥ 2	S < 1 R ≥ 1
Anidulafungin	Twofolds Serial Dilutions			0.06- 4 µg/ml	0.125- 8 µg/ml	0.125- 8 µg/ml
	Recommended Cutoff Value			S ≤ 0.5 R ≥ 1	S ≤ 4 R > 4	S ≤ 1 R > 1
Micafungin	Twofolds Serial Dilutions				0.5- 32 µg/ml	0.5- 32 µg/ml
	Recommended Cutoff Value				S ≤ 8 R > 8	S ≤ 4 R > 4

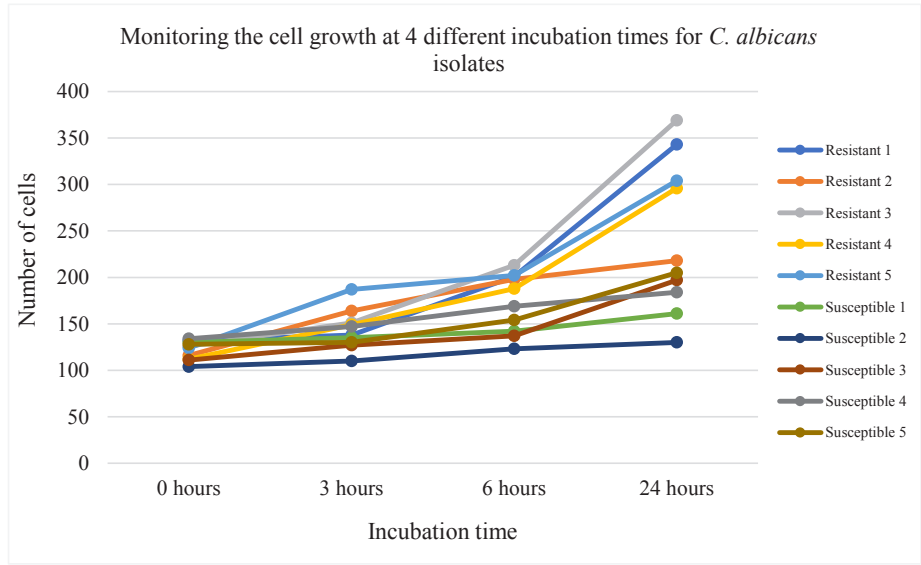
The first challenge to set up MBT ASTRA for yeasts was the variation in growth rates of these microorganisms. The *Candida* cell cycle usually takes between 1 and 2 h, which is longer than the usual cell cycle of fast growing bacteria of about 20 minutes (24, 25). It has been a challenge to set up MBT ASTRA for yeasts using only a few hours of incubation. To complete and reliably detect the variation in cell growth between the control and the samples containing different antifungal concentrations by MALDI-TOF MS, the cell densities should be between 2.40 × 10^8 and 1.10 × 10^{10} viable cell counts (VCCs) per ml, depending on the species (26).

Therefore, for every species, 5 susceptible and 5 resistant isolates were selected in the control set up, and then the number of cells was counted after 0 h, 3 h, 6 h, and 24 h incubation at 37°C in a ThermoMixer (brand) with shaking at 300 rpm. Cell counting was performed using 10 µl of the taken sample in a Neubauer hemocytometer (Marienfeld, Germany), and then the average of the cell growth was calculated for susceptible and resistant isolates (Total number = 10; 5 susceptible and 5 resistant). As an example, data acquired for *C. albicans* are shown in Figure 2 and Table 2. As shown in Figure 2 a and b, most of the isolates showed growth after 3h of incubation. However, the log phase was only observed at an incubation time of 6h for all isolates. Therefore, the MBT ASTRA method was set up for 6h of incubation time for all *Candida* spp.

Table 2. The number of *Candida albicans* cells counted after zero, three, six, and twenty-four hours incubation at 37°C in a ThermoMixer with shaking at 300 rpm.

	Strains	0 hours (Number of cells/ml)	3 hours (Number of cells/ml)	6 hours (Number of cells/ml)	24 hours (Number of cells/ml)
Susceptible strains	**CBS 8758**	13000	13500	14200	16100
	SCS 68225	10400	11000	12300	13000
	SCS 17181788	11100	12700	13700	19700
	AM_2003/0044	13400	14700	16900	18400
	PEG 10-91-43PEG	12800	13000	15400	20500
Resistant strains	**CLF-2**	13200	13800	20100	34300
	CLF-177	11600	16400	19800	21800
	CLF-283	12600	15100	21300	36900
	CLF-539	11200	14900	18800	29600
	B12_017053	12400	18700	20200	30400
Average		12200	14400	17300	24100

a



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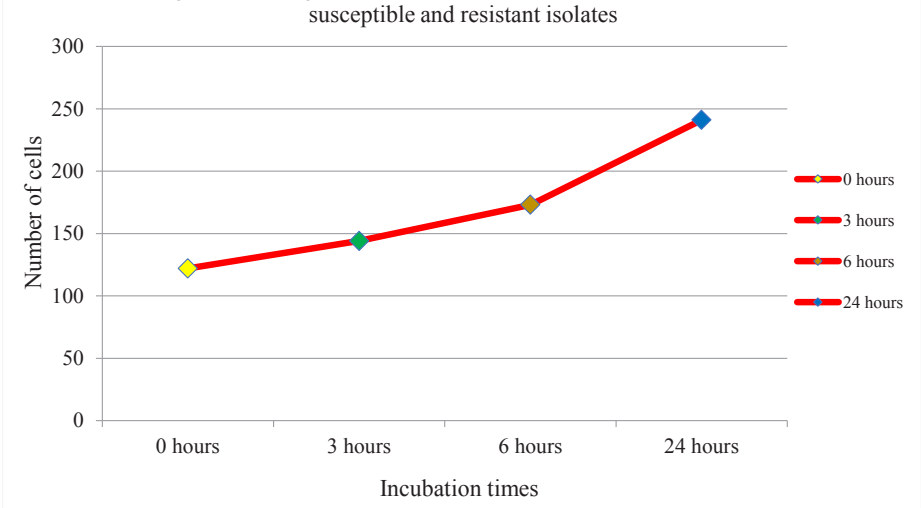


Figure 2. Cell numbers for *Candida albicans* isolates (resistant numbers = 5, susceptible numbers = 5) grown at four different incubation times (0, 3, 6, 24 h) (a), and their calculated average for both susceptible and resistant isolates (total numbers = 10 isolaes) (b).

The second challenge for the development of the MBT ASTRA protocol for yeasts was to find a way to capture the cells to perform the extraction of proteins after 6h of incubation. According to the few numbers of cells in the control as well as in the presence of antifungal drugs, using the standard extraction method for MALDI-TOF MS was not practical. Therefore, it was decided to use multiwell filter plates to have more capability to capture a low number of cells. There are a variety of multiwell filter plates with different volumes of the wells and filter pore sizes. Firstly, a multiwell filter plate 350 µl well and 0.22 µm-pore-size filter with a GH Polypro (GHP) membrane (Pall, USA) was used. The outcome was not as promising as expected. We found that due to the presence of a low number of cells in the setup, a larger volume of the cells had to be used for the extraction. Therefore, another multiwell filter plate was tested with a 1 ml well; 0.45 µm-pore-size filter with a GH Polypro (GHP) membrane (Pall, USA), and this worked out nicely. Five-hundred out of 600 µl of yeasts grown in the RMPI medium were used for the extraction.

The third step was to figure out the procedure of the protein extraction. Cells were collected after 5 min centrifugation at 4,000 × *g* for 5 min (5804 R centrifuge; Eppendorf, Germany). The collected cells were rinsed with H_2O to remove the RPMI 1640 medium as this interferes with the MALDI-TOF MS measuring. Different factors were tested, like the volume of H_2O, numbers of washing steps, and the times needed for centrifugation. Finally, the best outcome was observed after 2× rinsing with 200 µl of H_2O and centrifugation at 4,000 × *g* for 3 min. The same procedures were considered and tested for 75% ethanol, 70% formic acid (Merck, Germany), and 100% acetonitrile (ROTH, Germany). Eventually, after investigating different factors, one-time loading of 100 µl 75% ethanol for 3 min centrifugation at 4,000 × *g* was applied. The last step was to complete the protein extraction using 70% formic acid and 100% acetonitrile. The best outcome was achieved by adding 10 µl of each and then 3 min centrifugation at 4,000 × *g*. This last step was repeated twice to have optimal and maximal cell

lysis and extraction of proteins. The lysate acquired from each setup was spotted twice onto a polished steel target plate (Bruker Daltonik) and dried at room temperature. The spots were overlaid with 1 µl MALDI matrix (10 mg/ml of α-cyano-4-hydroxy-cinnamic acid [α-HCCA] in 50% acetonitrile–2.5% trifluoroacetic acid; Bruker Daltonik) containing MBT ASTRA standard II (proprietary of Bruker Daltonik). The target was introduced into the MALDI-TOF MS and spectra were acquired with a Microflex LT/SH mass spectrometer (Bruker Daltonik) in a mass range between 2,000 and 20,000 Da and calibrated with a bacterial test standard (BTS; Bruker Daltonik). The MBT ASTRA method used initial and maximal laser powers between 30- 40% with a signal to noise threshold of 2. Forty shots were used at laser spot and in total 480 shots were accumulated for every spot on the target. A maximum number of 300 peaks at the minimum intensity threshold of 400 were considered for analysis. Figure 3 shows the difference between the standard protein extraction method and the extraction that used multiwell filter plates (1ml well, 0.45µm GHP, PALL, USA). The number and intensity of peaks are significantly higher by the MBT ASTRA extraction method when compared to the standard extraction method (Figure 3).

MBT ASTRA standard II: The final and the most important part to set up MBT ASTRA for clinically relevant yeasts was to choose the proper internal standard. The MBT ASTRA standard is a protein that can be detected by MALDI-TOF MS and preferably has a high stability. This protein is added to the matrix to perform further analysis by MS ASTRA prototype software (proprietary of Bruker Daltonik, Germany). MBT ASTRA standard II may suppress *Candida* peaks and has an impact on the outcome that results from the MS ASTRA prototype software. Therefore, it is crucial to use a standard that has preferably the peak(s) outside of the area in which the most predominant peaks of the respective microorganism occur.

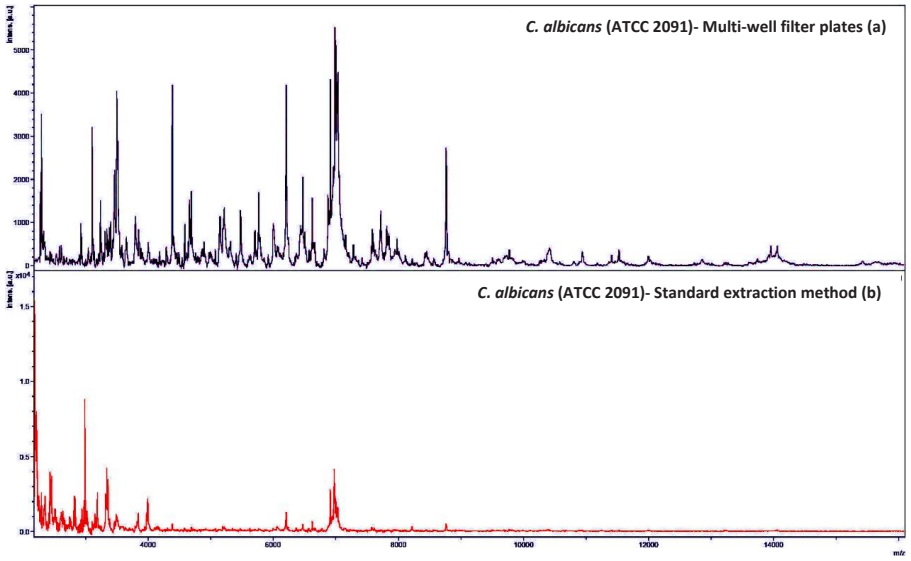


Figure 3. Comparison between acquired spectra of the same *C. albicans* isolate (ATCC 2091) extracted by multiwell filter plates (1ml well, 0.45µm GHP) (a), and by the standard method in use for MALDI-TOF MS (b). A suspension of 0.5 McFarland of the *C. albicans* reference isolate was prepared and diluted 1:20 in 600 µl of RPMI 1640. Cells were incubated for 6h and then extracted using two methods. The number and intensity of the peaks acquired by the filter plate are significantly higher than the standard extraction method due to the low number of cells present.

Furthermore, the amount of the standard added to the matrix must be adjusted to the intensity of the peaks in the control of the respective species, as this differs from one species to another. This optimization is performed after the analysis of the acquired data by MS ASTRA prototype software. Using this software, the growth rate of the control of each species was assessed and according to the intensity of the acquired peaks, the amount of the standard determined. One of the achievements of this Ph.D. project was to find a novel standard for the MBT ASTRA method for *Candida* species. This standard is different from the one used for the analysis of bacteria. This protein is now called MBT ASTRA standard II and it is confidential to the Bruker

company, but it is commercially available for research laboratories. Figure 4 shows the differentiation between the spectrum profile of a *C. albicans* isolate using the control with and without the standard.

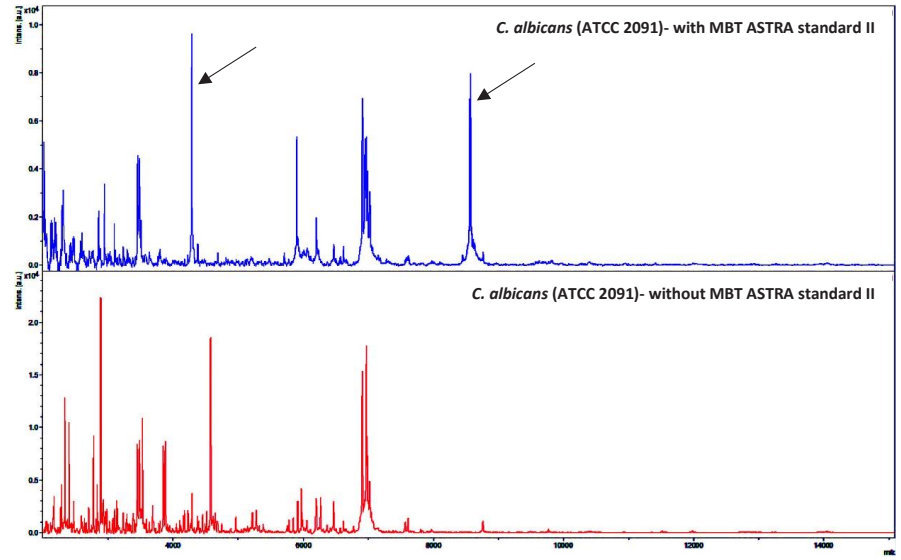
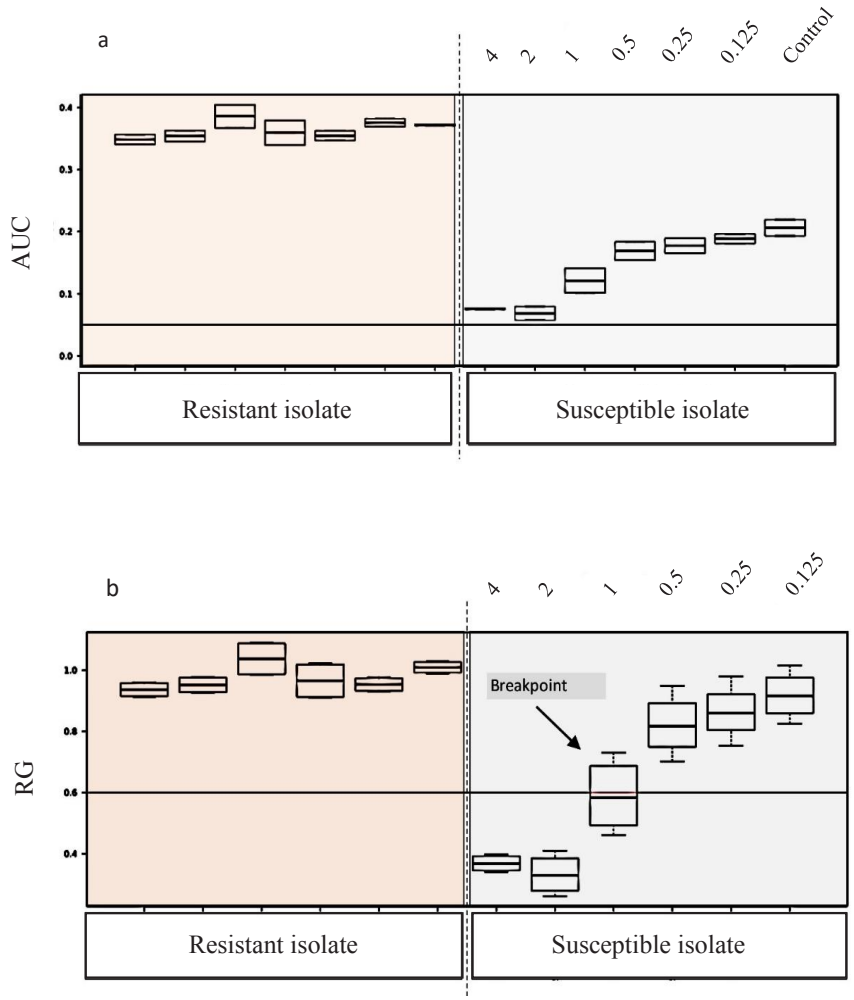


Figure 4. *Candida albicans* MALDI-TOF MS profiles in the control setup with and without MBT ASTRA standard II. Marked peaks belong to the standard and their high intensity may suppress some few peaks around them. These peaks are used to normalize the acquired AUC for antifungal setup and control.

MS ASTRA prototype software: This prototype software has already been described (2, 14, 15). After defining the RG threshold, it is used to determine the unique cutoff value for each strain/species and the respective antifungal. The cutoff value calculated by MS ASTRA is compared to the MICs µg/ml as set by the CLSI and EUCAST methods. Accordingly, strains that show an RG above the determined threshold are considered as resistant and those with an RG beneath this threshold are considered susceptible. In addition, only isolates that could pass

the growth control cutoff were considered for further analysis by the MS ASTRA prototype. Like the EUCAST method that analyzes the susceptibility of an isolate based on the number of cells by the spectrophotometer, here the number and intensity of the expressed proteins for each setup were evaluated. The expressed proteins are associated with the growth of cells in each setup that are compared to the control. The MS ASTRA prototype software function and the way of evaluating data are briefly described in figure 5. As an example, the analysis of *C. albicans* isolates (susceptible, N=1, resistant, N=1) against caspofungin is shown in figure 5.



c

	Strains	0 hours (Number of cells/ml)	3 hours (Number of cells/ml)	6 hours (Number of cells/ml)	24 hours (Number of cells/ml)
Susceptible strains	**CBS 8758**	13000	13500	14200	16100
	SCS 68225	10400	11000	12300	13000
	SCS 17181788	11100	12700	13700	19700
	AM_2003/0044	13400	14700	16900	18400
	PEG 10-91-43PEG	12800	13000	15400	20500
Resistant strains	**CLF-2**	13200	13800	20100	34300
	CLF-177	11600	16400	19800	21800
	CLF-283	12600	15100	21300	36900
	CLF-539	11200	14900	18800	29600
	B12_017053	12400	18700	20200	30400
Average		12200	14400	17300	24100

d

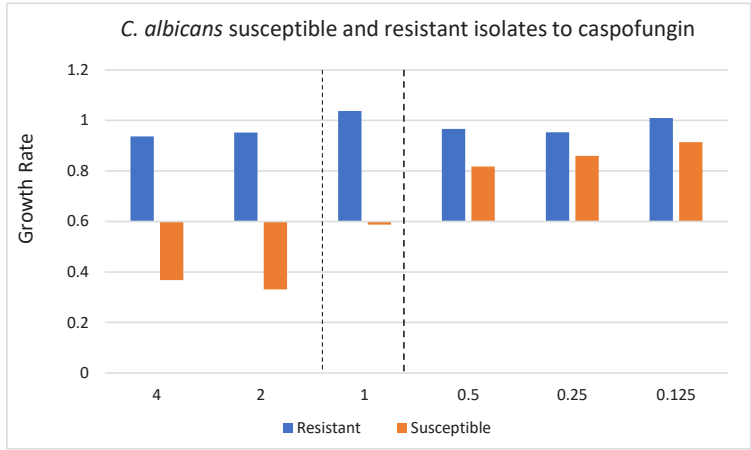


Figure 5. An example of the MBT ASTRA software prototype function to analyze acquired MALDI-TOF MS spectra based on the cell growth and evaluation of the proteins. Firstly, a boxplot is made based on AUC for the control and in the presence of different caspofungin concentrations (a). Then, after normalization of the peaks to the standard, the relative growth (RG) threshold for each isolate is calculated. The RG threshold is used to determine the unique cutoff value for each species against the respective antibiotic/antifungal (b). In this example, the RG threshold is set to 0.6, and accordingly, the cutoff value is determined at 1µg/ml. Afterward, MS ASTRA prototype provides the value of whole measured proteins (c), which are used for further analysis to evaluate the final breakpoint comparing susceptible and resistant isolates (d).

The susceptibility of every isolate is firstly determined by either the CLSI or the EUCAST methods to find out the MICs µg/ml for every isolate. Next, the isolates are analyzed by the MBT ASTRA method. Primary evaluation of the growth of the control and each antifungal setup is performed. Then, based on the background information regarding the susceptibility of each isolate, the best RG threshold is determined to distinguish accurately between susceptible and resistant isolates. First, the cutoff value is defined for a few numbers of isolates of a *Candida* species, then it will be expanded to the larger sample size to obtain a final evaluation. Afterwards, the final cutoff value found by MS ASTRA is compared to that obtained by the microdilution reference methods. MBT ASTRA optimization initially used colonies from SDA plates and was subsequently tested using spiked broth blood culture; subsequently, it was tested using patient´s blood samples. This method was used for the first time in a routine clinical laboratory for two weeks. Meanwhile, six patients' blood samples infected with *C. glabrata* were collected and tested by MBT ASTRA for susceptibility to anidulafungin. These isolates were detected before as susceptible when they had been analyzed by disc diffusion. MBT ASTRA detected 5 susceptible isolates correctly and one susceptible isolate was detected as resistant. Although disc diffusion is not a recommended method for fungi, further analysis of gene sequencing and/or use of microdilution methods illustrated the discrepancy between the two methods (Figure 6).

The cutoff value might change between one or two serial dilutions starting from SDA if compared to data obtained using the patient´s blood samples. Taken together, this study illustrated a variety of zero to twofold serial dilutions between the MBT ASTRA cutoff values and the MIC µg/ml of the microdilution methods for *C. auris, C. albicans*, and *C. glabrata* against echinocandins (Table 1, see also Table 2- Chapter 1). However, since MBT ASTRA is a novel method, introducing a new cutoff value (breakpoint) is feasible for this method and needed for future use in routine clinical laboratories.

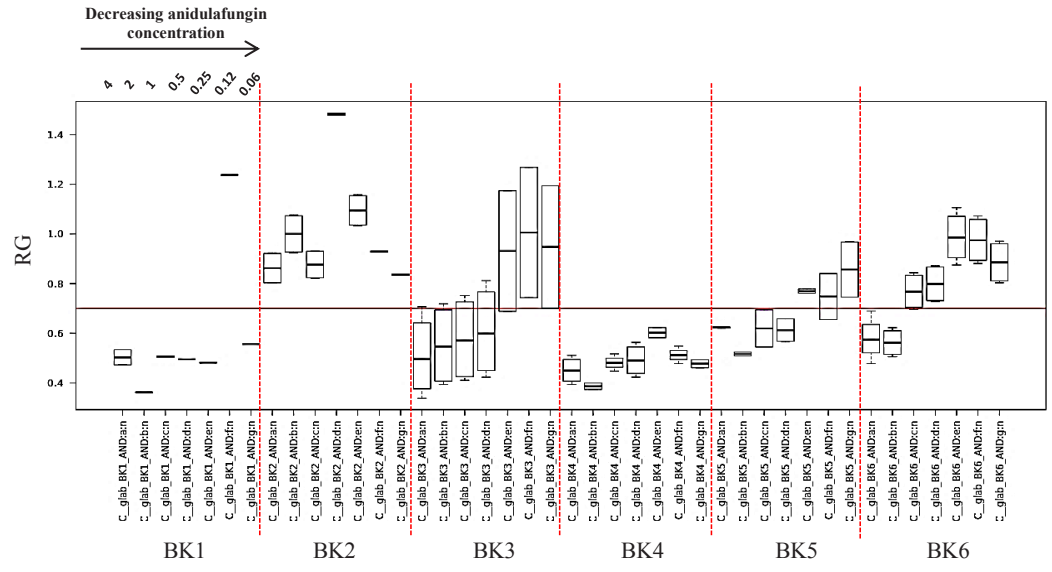


Figure 6. Performance of MBT ASTRA directly on patient's blood samples infected with *Candida glabrata*. Six samples (BK1-BK6) were tested against anidulafungin by disc diffusion and were detected as susceptible. Then, they were analyzed by the MBT ASTRA. The same anidulafungin concentrations showed for BK1 was used for all isolates (from 0.6 µg/ml up to 4 µg/ml). Five out of six susceptible isolates were detected correctly by the MBT ASTRA. Only one isolate, BK2, was wrongly detected as resistant.

Contrary to the microdilution methods, MBT ASTRA is not able to detect intermediate isolates yet. Further analysis using a larger sample size may show the future applicability of this method to also detect intermediate isolates. This is important, as in hospitals intermediate isolates are considered resistant to get proper treatment management. It is noteworthy that EUCAST has recommended using a high dosage of a drug or a physical injection of antibiotic/antifungal at the site of infection to treat intermediate isolates. Therefore, all intermediate isolates as obtained by the microdilution methods are categorized as resistant by the MBT ASTRA assay to allow appropriate patient management in the future. Misclassification of intermediate

isolates as susceptible by MBT ASTRA reduced the sensitivity of the method and interfere with proper antifungal therapy. This misclassification was rarely observed when MBT ASTRA was tested for anidulafungin on broth blood cultures spiked with *C. glabrata*. One possible reason for this may be related to the method used for the blood lysis that probably changed the *C. glabrata* cell wall structure and subsequently impacted the targets of anidulafungin located in the cell wall (17). However, excluding this disadvantage, the possibility of this method to detect isolates resistant to echinocandins rapidly in about 6h requires further optimization and standardization before it can be introduced into clinical laboratories. In summary, to obtain an optimal result by using the MBT ASTRA method several parameters, such as sample preparation, lysis of blood cells, protein extraction method, proper MBT ASTRA standard component, and a precise data analysis prototype need to be optimized. A brief protocol of the MBT ASTRA method that we developed is described in figure 7.

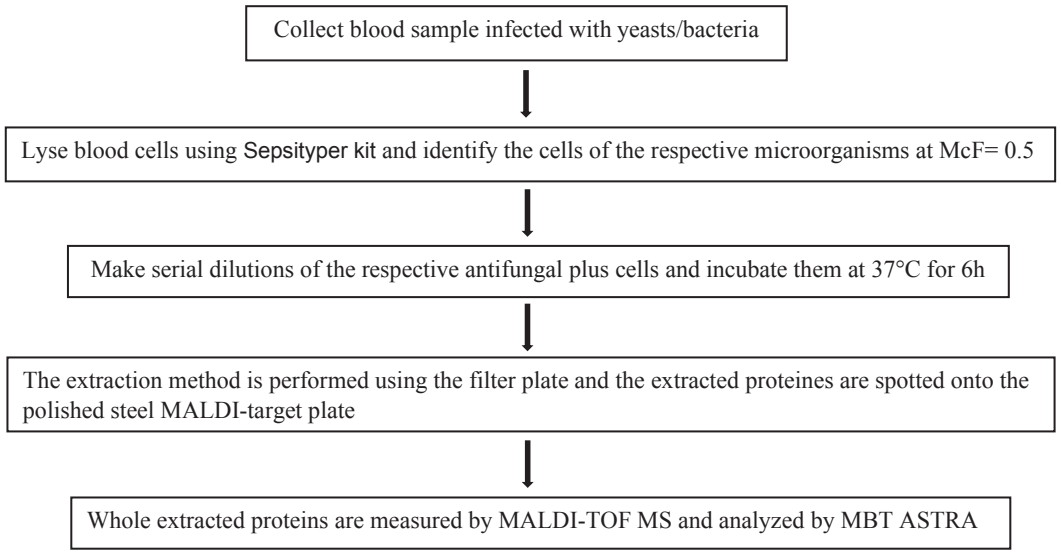


Figure 7. Stepwise description of the MBT ASTRA method.

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Chapter 3

Proof of Concept for MBT ASTRA, a rapid Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS)-based method to detect caspofungin resistance in *Candida albicans* and *Candida glabrata*

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Abstract

Candidemia caused by *Candida albicans* and *Candida glabrata* is constantly increasing, accompanied by a rising use of the few available antifungals. The widespread use of echinocandins and azoles for treatment of invasive candidemia has enhanced the development of antifungal resistance resulting in an increasing health care problem. Hence, the rapid detection of resistant strains is required. This study aimed to evaluate the detection of *C. albicans* and *C. glabrata* strains resistant against caspofungin by MALDI Biotyper antibiotic susceptibility test- rapid assay (MBT ASTRA). This novel semi-quantitative technique facilitates the detection of caspofungin-resistant strains within 6 hours. MBT ASTRA results were compared to the data obtained by the Clinical and Laboratory Standards Institute guidelines (CLSI). Clinical isolates of *C. albicans* (n = 58) and *C. glabrata* (n = 57) were analysed by MBT ASTRA and CLSI microdilution. CLSI antifungal susceptibility test against caspofungin classified *C. albicans* isolates into 36 susceptible and 22 resistant strains, and *C. glabrata* isolates into 5 susceptible, 33 resistant and 19 intermediate, respectively. For *C. albicans*, the comparison of MBT ASTRA and the CLSI method revealed an excellent categorical agreement of 100%. Sensitivity and specificity of 94% and 80% between MBT ASTRA and CLSI were detected for *C. glabrata* strains, based on categorical agreement, respectively. In conclusion, the results obtained by MBT ASTRA indicated that this is a very promising approach for the rapid detection of candida isolates resistant to caspofungin.

Keywords: *Candida* spp., MALDI-TOF MS, antifungal susceptibility testing

Introduction

Invasive fungal diseases are a prevalent life-threatening complication in patients that are immune-suppressed, received chemotherapy, suffering from haematological disorders, transplanted and ICU admitted (1, 2). Several *Candida* species cause opportunistic fungal infections and are responsible for 70–90% of all invasive fungal infections. Among *Candida* species, *Candida albicans* is the major species isolated from patients (1, 3) followed by *Candida glabrata* (2, 4). *C. albicans* is estimated to cause mortality rates as high as 45% which can be due to a lack of rapid diagnostic methods and/or inappropriate antifungal treatment (5). The incidence of candidemia caused by *C. albicans* and *C. glabrata* is globally increasing (5), which is corresponding to application of only a few classes of antifungals (6, 7). Therefore, there is an apprehension that the efficacy of the major antifungals will be reduced resulting in limited therapeutic options in future (6, 8). Caspofungin belonging to the class of echinocandin drugs is one of the modern lipo-peptide antifungals and is often used as initial therapy against invasive candidiasis. This drug inhibits the β-1,3 glucan synthase that is essential to produce β-1,3 glucans, one of the important components of the fungal cell wall. In *C. albicans*, resistance to caspofungin is mostly a consequence of mutations in two hot spot regions in the *FKS1* gene. Additionally, Roman et al. (2016) have shown that CAT1 overproduction could increase the minimum inhibitory concentration to caspofungin twofold. Another study demonstrated that negative regulation of one copy of chromosome 5 (Ch5) gene may be associated with echinocandin tolerance in *C. albicans* (9–13). While resistance to caspofungin in *C. glabrata* is related to mutations in the *FKS1* and the *FKS2* genes (9, 14, 15).

Fast and reliable detection of *C. albicans* and *C. glabrata* isolates resistant against caspofungin is a major prerequisite for appropriate patient management. Despite advantages of conventional susceptibility tests such as microdilution according to CLSI or to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guideline, these standard

methods require long incubation time that has received considerable critical attention in clinical laboratories. Recent trends in the substitution of standard microdilution methods by commercial approaches promoted antifungal susceptibility tests (AFST) that obtained reliable results within somewhat shortened incubation times (16–19).

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) is an easy, rapid, cost effective and high throughput technology with outstanding accuracy in species identification (20, 21). Subsequent to the identification of microorganisms, this technique has recently been applied for antimicrobial susceptibility testing (20, 22). Previously, the MALDI Biotyper antibiotic susceptibility test rapid assay (MBT ASTRA) has been described as a novel semi-quantitative technique for susceptibility testing in bacteria (23). This approach is a phenotypic assay comparing the cell growth in the presence of an antibiotic to the growth in a control setup without antibiotic. Due to the high sensitivity of MALDI-TOF MS, differences between susceptible and resistant strain are detectable within a few hours (3-5h for bacteria) by semi-quantitative analysis of the acquired mass spectra. In this study, MBT ASTRA was optimized and applied to susceptibility testing in yeasts such as *C. albicans* and *C. glabrata*.

Materials and methods

Strains. Clinical isolates of *C. albicans* (n= 58) and *C. glabrata* (n= 57) obtained from three different clinical origins; Medical University of Innsbruck, Austria, Westerdijk Fungal Biodiversity Institute, The Netherlands and Max von Pettenkofer-Institut, Germany were analysed. Species were confirmed by MALDI Biotyper (Bruker Daltonik GmbH, Germany). CLSI antifungal susceptibility test against caspofungin classified *C. albicans* strains into 36 susceptible and 22 resistant strains, and *C. glabrata* isolates into 5 susceptible, 33 resistant and 19 intermediate strains. Isolates stored at -80°C were directly cultivated on Sabouraud Dextrose

Agar (SDA) before use overnight at 35°C. The reference organisms *C. parapsilosis* ATCC 22019 and *C. krusei* (*Pichia kudriavzevii*) ATCC 6258 recommended by CLSI were used as quality control strains for susceptibility testing by microdilution (CLSI). *C. albicans* ATCC 64548 and *C. albicans* ATCC 64550 recommended by EUCAST as quality control strains for caspofungin testing were used in both, microdilution and MBT ASTRA (24, 25).

Antifungal susceptibility testing. *In vitro* antifungal susceptibility test was performed by classical microdilution employing twofold serial dilutions of caspofungin (Sigma-Aldrich, Germany) according to the current CLSI guideline M60 (November 2017) (24). Breakpoints were applied according to the current CLSI guideline M60 (November 2017) for *C. albicans* (clinical breakpoints: susceptible, ≤0.25 µg/ml; resistant, ≥1 µg/ml and intermediate= 0.5 µg/ml) and *C. glabrata* (clinical breakpoints: susceptible, ≤0.125 µg/ml; resistant, ≥0.5 µg/ml and intermediate = 0.25 µg/ml) (24). Slow growing *Candida* strains that could not be visually evaluated, were analyzed by microplate reader (CLARIOstar/BMG LABTECH, Germany) in flat bottom microplates measured at 450 nm after 48h incubation at 37°C, without shaking.

MBT ASTRA. Cell suspension of McF 0.5 (Grant-bio-DEN-1, England) prepared from fresh overnight cultures of individual isolates was diluted 1:20 into RPMI 1640 medium (Sigma-Aldrich, Germany) supplemented with a final concentration of 0.165 Mol/L morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich, Germany) and 2% glucose (Sigma-Aldrich, Germany) and adjusted to pH 7.0 (25). Cells were incubated at 37°C for 0h, 3h, 6h, and 24h. Ten microliters of the cell suspension were used at each time point for cell counting in a Neubauer hemocytometer (Marienfeld, Germany). The average of four replicates was calculated.

The same suspension used for cell counting was also applied for the MALDI-TOF MS based resistance assay. Twofold serial dilutions of caspofungin were prepared ranging from 0.125 up to 4 µg/ml plus a control without antifungal containing 0.5-2.5×10^5 cells/L in a volume of 600 µl (25). Incubation was performed at 37°C for 6h in a ThermoMixer (Eppendorf, Germany) for 4h without agitation followed by 2h under agitation at 300 rpm. Multi-well filter plates (1ml well, 0.45µm GHP, PALL, United States) were employed to collect the cells after incubation by centrifugation at 4000×g for 5min (Eppendorf, Centrifuge 5804 R, Germany). The cells were rinsed twice with 200 µl sterile deionized water and once with 100 µl 75% ethanol. Cell lysis was performed according to the MALDI Biotyper standard protocol (26) using 10.5 µl 70% formic acid (Merck, Germany) and 10.5 µl 100% acetonitrile (Roth, Germany) directly on the filter. Cell lysis using formic acid and acetonitrile was repeated twice. For MALDI-TOF MS measurements, 1 µl lysate of each set up was spotted in duplicate onto a polished steel target plate (Bruker Daltonik GmbH, Germany) and overlaid with 1 µl MALDI matrix (10 mg/ml of α -cyano-4-hydroxy-cinnamic acid [α-HCCA] in 50% acetonitrile–2.5% trifluoroacetic acid; Bruker Daltonik) containing the MBT ASTRA Standard II (Bruker Daltonik). MALDI-TOF MS spectra were acquired on a Microflex LT/SH mass spectrometer (Bruker Daltonik) calibrated with a bacterial test standard (BTS; Bruker Daltonik) in the mass range between 2,000 and 20,000 Da (23).

Data analysis. Acquired spectra were analyzed by MS ASTRA prototype software written in "R" according to the procedure described by Lange *et al.* (2014) (23, 27, 28) resulting in the AUC (area under the curve) for each incubation setup. The relative growth (RG) was individually calculated for each concentration of caspofungin determining the ratio of the AUC of the antifungal setup to the AUC of the control:

$RG = (\text{AUC}_{\text{RPMI+caspofungin}})/(\text{AUC}_{\text{RPMI}})$

The RG cut-off was set to 0.6 RG units. Accordingly, strains showing a RG above this threshold were considered as resistant and those revealing a RG below this cutoff were considered as susceptible. Further development will be necessary to improve this software and provide it to customers. CLSI was used as a gold standard and sensitivity and specificity were calculated for MBT ASTRA based on this method.

Results

Optimization of MBT ASTRA. MBT ASTRA has been originally developed to detect resistance of bacterial strains against different antibiotics within 1 to 3h. Adaptation of this protocol for the analysis of yeasts required the definition of the minimal and sufficient incubation time in which both species showed a significant growth. A collection of 20 strains (10 *C. albicans* and 10 *C. glabrata*), including 5 susceptible and 5 resistant strains for each species, was selected for counting of the yeast cells as described above. Monitoring cell growth revealed that *C. albicans* strains resistant to caspofungin showed a faster growth compared to susceptible strains (data were not shown). The growth rates of susceptible and resistant *C. glabrata* strains were approximately identical. The results demonstrated that detectable cell growth started after 3h for both species, but a significant growth was observed between 5 and 6h for all strains. An incubation time of 6h was defined for MBT ASTRA fulfilling the growth requirements for *C. albicans* and *C. glabrata* strains.

A total of 16 strains, including four susceptible isolates and four isolates resistant to caspofungin, of each species were used to optimize MBT ASTRA. Before testing, the *FKS1* and *FKS2* genes were sequenced for these isolates as described elsewhere (Table 1) (29) and identification was performed by MALDI Biotyper to confirm species identity. MBT ASTRA

was performed for these strains as described above. The acquired spectra were processed by MBT ASTRA prototype software calculating first the area under the curve (AUC) that is directly corresponding to the growth of this strain within the respective setup. This analysis revealed that the growth (AUC) varied for each strain and each analytical setup depending on the individual growth capacity and on the resistance status of the strain. The detailed analysis of the data demonstrated that it was necessary to introduce a threshold for the minimum growth of the control setups without antibiotic to achieve reliable categorization of the strains. For each strain passing this threshold, the relative growth was calculated as a measure of the growth of the respective strain in the presence of antifungal. A cut-off value (RG) was defined at 0.6 for both species. The titration experiments revealed that each strain changed its behavior from "growth" to "no growth" at an individual caspofungin concentration. The RGs of all *C. albicans* strains previously characterized as susceptible by gene sequencing was below the threshold of 0.6 (RG <0.6) at caspofungin concentration of 1 µg/ml or below. In contrast, the RGs of *C. albicans* strains that had been characterized as resistant by gene sequencing showed this switch only at caspofungin concentrations above 1 µg/ml (Figure. 1 A). For *C. glabrata*, the same behavior was observed with the difference that the caspofungin breakpoint concentration for susceptible strains was at 0.5 µg/ml or below and for resistant strains above 0.5 µg/ml (Figure 1 B). These results imply that MBT ASTRA specific breakpoint concentrations were required for categorization of the strains. Compared to the CLSI breakpoints these novel MBT ASTRA breakpoint concentrations were one dilution step above the CLSI breakpoints.

Reproducibility of MBT ASTRA. Reproducibility of this novel approach was tested on three different days by analysis of twenty isolates including five susceptible and five resistant strains randomly selected of each species (Figure. 2).

Table 1. *FKS1* and *FKS2* genes sequencing results of *C. albicans* and *C. glabrata* strains.

		Genotype	
Species	**Strains**	*FKS1*	*FKS2*
***C. albicans* (8)**	ATCC 64548	WT	
	ATCC 64550	WT	
	ATCC 24433	WT	
	CBS_8758	WT	
	CLF_11	S645P	
	CLF_41	F641S	
	CLF_52	S645Y	
	CLF_82	P649H	
***C. glabrata* (8)**	ATCC 2001		WT
	CBS_1518		WT
	CBS_2175		WT
	11876		WT
	CLF_4		*FKS*2p-F659S
	CLF_24		S629P
	CLF_34		D632G
	CLF_83		*FKS*2p-S663F

For *C. albicans*, agreement between these five individual experiments was observed for all susceptible strains showing a significant relative growth reduction below the RG threshold of 0.6 at caspofungin concentrations similar or below 1 µg/ml. Although the absolute values varied, the strains were correctly categorized as susceptible (Figure. 2 A) (supplementary 1; Figure 1 A-D). For resistant *C. albicans* strains, four strains were categorized as resistant in all three analyses (Figure. 2 B) (supplementary 1; Figure 1 E, G, H). Only one strain had a relative growth reduction at a caspofungin concentration of 1 µg/ml and showed an RG value slightly below the RG cut- off value and thereby, it was categorized as susceptible in one experiment (See supplementary 1; Figure 1 F). Surprisingly, the growth rate of this strain increased at a concentration 2 µg/ml again. For *C. glabrata*, four susceptible strains were constantly

categorized as susceptible in all three different experiments (Figure. 2 C) (supplementary 1; Figure 1 J, K, L). One susceptible strain showed a variable behaviour within the three different experiments (supplementary 1; Figure 1 I). In contrast, all five resistant strains were accurately detected (Figure. 2 D) (Supplementary 1; Figure 1 M-P). Intermediate *C. glabrata* strains revealed variant results pending between susceptible and resistant categorization (See supplementary 1; Figure 1 Q-S).

Analysis of clinical isolates by MBT ASTRA. After assay optimization, 115 clinical isolates were analyzed by MBT ASTRA and MALDI Biotyper according to the protocol previously described. The caspofungin concentration resulting in an RG value similar or below 0.6 was taken as individual minimal inhibitory concentration of each strain. Figure 3 represents the comparison of the CLSI derived MICs and the MBT ASTRA derived MICs. For *C. albicans*, complete agreement between both approaches was observed. All isolates that were categorized as susceptible by microdilution were also detected as susceptible by MBT ASTRA and all strains categorized as resistant by microdilution were found to be resistant by MBT ASTRA.

No intermediate strains were detected by microdilution and MBT ASTRA. In total, 29 susceptible and 22 resistant *C. albicans* strains were correctly categorized by MBT ASTRA within 6h (Figure 3 A, Table 2). Seven strains showed insufficient growth in the control set up and were excluded from the further evaluation resulting in a validity of the MBT-ASTRA approach of 88%. Sensitivity and specificity of MBT ASTRA were 100% excluding the seven slow growing strains for *C. albicans* compared to the CLSI microdilution method (Table 4).

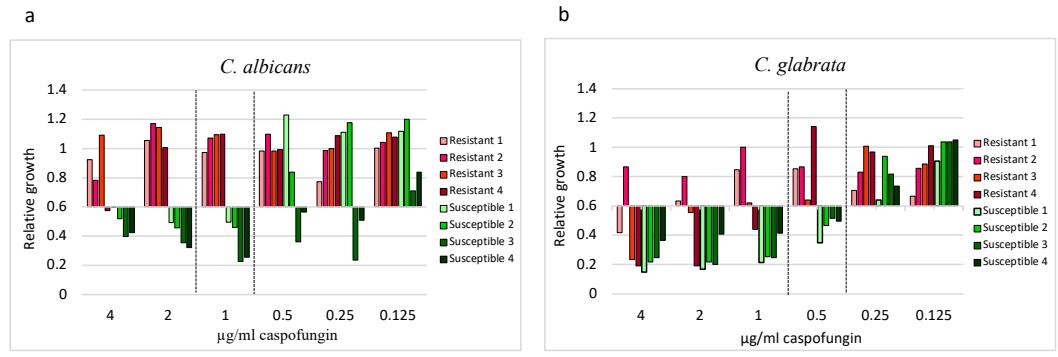


Figure 1. Optimization of MBT ASTRA for susceptibility testing of yeasts. Relative growth values of a twofold serial dilution of caspofungin in the range between 0.125 to 4 µg/ml for *Candida albicans* (a) and *Candida glabrata* (b) derived by MBT ASTRA. An MBT ASTRA caspofungin breakpoint was defined for *C. albicans* (a) and *C. glabrata* (b), respectively. *C. albicans* strains with MICs above 1 µg/ml caspofungin were categorized as resistant (number 1-4) and strains with MICs similar or below 1 µg/ml were categorized as susceptible (number 5-8) (a). For *C. glabrata*, strains with MICs above 0.5 µg/ml caspofungin were categorized as resistant (number 1-4) and strains with MICs similar or below 0.5 µg/ml caspofungin were categorized as susceptible (number 5-8).

For *C. glabrata*, 31 of 33 strains categorized as resistant by microdilution were also found as resistant by MBT ASTRA. Four of five susceptible strains were successfully detected by MBT ASTRA. Nineteen strains were categorized as intermediate by microdilution. Overall, strains showing intermediate behaviour in the microdilution were considered as resistant. Eighteen of these strains were categorized as resistant by MBT ASTRA that did not represent an intermediate category. Only one intermediate strain was classified as susceptible (Figure 4 B, Table 3). Sensitivity, specificity and validity of MBT ASTRA compared to microdilution method were calculated 94%, 80% and 95%, respectively (Table 4).

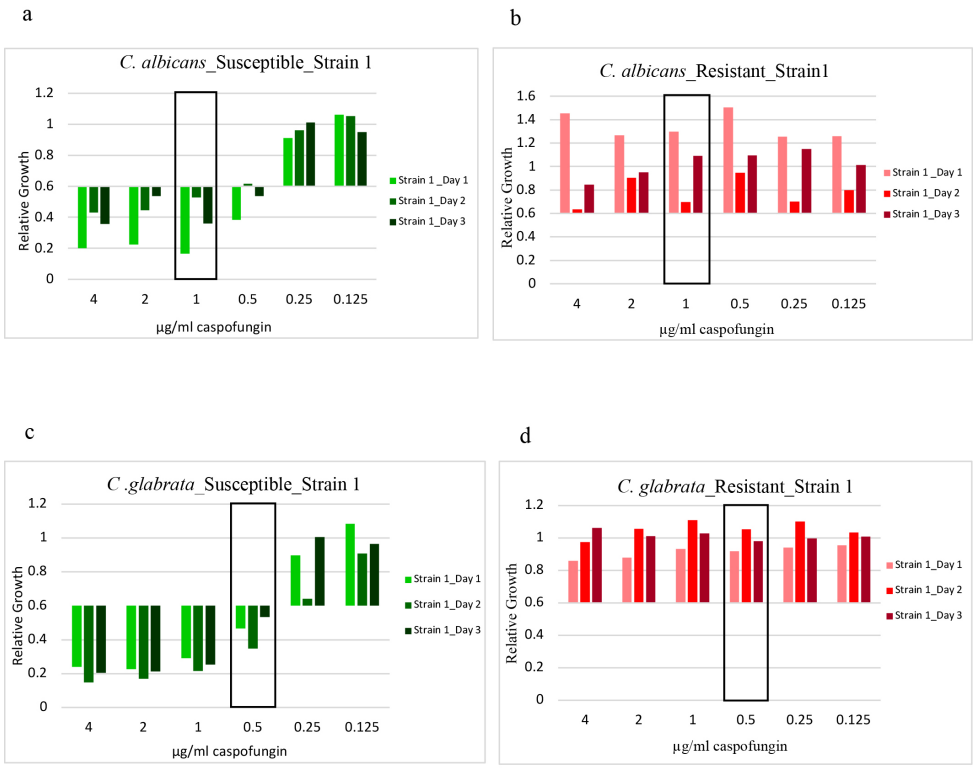
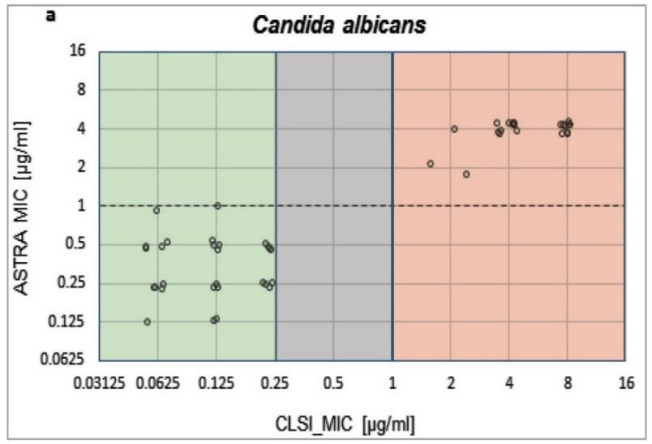


Figure 2. Example of the reproducibility of the MBT ASTRA. Relative growth values were obtained by two-fold serial dilutions of caspofungin for *Candida albicans* (susceptible (a), resistant (b)) and *Candida glabrata* (susceptible (c), resistant (d)) on three different days. *C. albicans* and *C. glabrata* revealed constant results at the breakpoint concentration (boxes) for the different experiments.



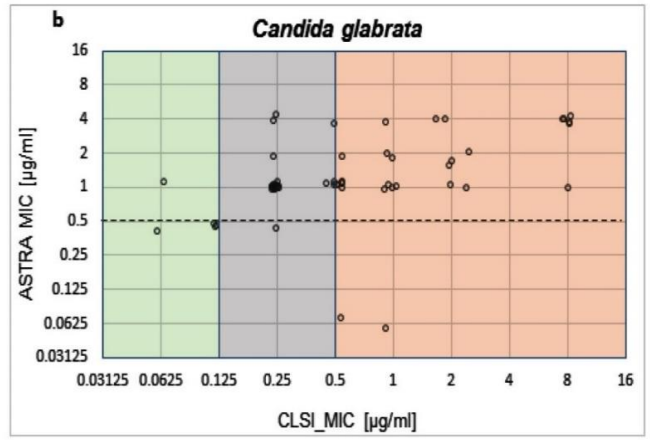


Figure 3. Comparison of MBT ASTRA and microdilution results of 51 clinical *Candida albicans* isolates and 57 clinical *Candida glabrata* isolates. For each strain, the MIC obtained by microdilution (*x* axis) was plotted against the MIC value derived by MBT ASTRA (*y* axis). The colored boxes indicate the MIC ranges according to CLSI; green: susceptible, gray: intermediate, red: resistant. The horizontal dashed line indicates the breakpoint concentration defined for MBT ASTRA MIC. For *C. albicans*, total agreement was observed between both approaches (a). For *C. glabrata,* concordant results were observed for 53 strains by both methods. Only one susceptible strain was detected as resistant and two resistant isolates were misclassified as susceptible strains by MBT ASTRA (b).

Table 2. *In vitro* caspofungin susceptibility test using CLSI and MBT ASTRA for 58 isolates of *Candida albicans*. Strains number 1-22 are resistant and 23-58 are susceptible.

C. albicans strains	CLSI MIC (Visually) [µg/ml]		MBT ASTRA MIC [µg/ml]
	24h	48h	6h
1	8	8	4
2	8	>8	4
3	2	4	4
4	4	8	4
5	?	4	4
6	?	2	2
7	?	2	2
8	?	2	4
9	> 8	>8	4
10	> 8	> 8	4
11	> 8	> 8	4
12	0.5	4	4
13	1	4	4
14	> 8	> 8	4
15	> 8	> 8	4
16	4	4	4
17	2	4	4
18	2	4	4
19	4	4	4
20	4	4	4
21	8	8	4
22	4	4	4
23	0.06	0.06	≤ 0.25
24	0.06	0.06	≤ 0.25
25	0.06	0.125	≤ 0.5
26	0.125	0.25	≤ 0.125
27	0.06	0.06	≤ 1
28	0.06	0.125	≤ 0.5
29	0.06	0.06	≤ 0.125
30	0.125	0.5	≤ 0.25
31	0.25	0.25	≤ 0.5
32	0.25	0.25	≤ 0.5
33	0.25	0.25	≤ 0.5
34	0.25	0.25	≤ 0.25
35	0.25	0.5	≤ 0.25
36	0.125	0.5	≤ 0.25
37	0.25	0.5	≤ 0.5
38	0.25	0.5	≤ 0.25
39	0.125	0.25	≤ 1
40	0.125	0.25	≤ 0.5
41	0.125	0.25	≤0.5
42	0.125	0.25	≤ 0.5
43	0.06	0.06	≤ 0.5
44	0.125	0.06	≤ 0.25
45	NA	0.5*	≤ 0.5
46	0.06	0.06	≤ 0.25
47	0.25	0.125	≤ 0.25
48	0.125	0.25	≤ 0.125
49	0.06	0.06	≤ 0.5
50	0.125	0.06	≤0.5
51	0.06	0.06	≤ 0.25
52	0.125	0.125	NA
53	0.125	0.25	NA
54	0.25	0.25	NA
55	0.06	0.125	NA
56	0.25	0.25	NA
57	0.125	0.25	NA
58	0.06	0.06	NA
ATCC 64548	0.125	0.125	≤1
ATCC 64550	0.25	0.25	≤1
ATCC 22019 ×	1	2	×
ATCC 6258 ×	1	1	×

Table 3. *In vitro* caspofungin susceptibility test using CLSI and MBT ASTRA for 57 isolates of *Candida glabrata*. Strains number 1-33 are resistant, 34-52 are intermediate and 53-57 are susceptible.

C. glabrata strains	CLSI MIC (Visually) [µg/ml]		MBT ASTRA MIC [µg/ml]
	48h	72h	6h
1	8	8	4
2	2	2	4
3	1	1	4
4	1	1	2
5	4	8	4
6	2	2	2
7	NA	2	4
8	NA	2	1
9	2	2	2
10	NA	2	2
11	0.5	0.5	1
12	0.5	0.5	1
13	0.5	1	1
14	0.5	0.5	1
15	0.5	1	1
16	0.5	0.5	4
17	>8	>8	4
18	>8	>8	4
19	0.5	0.5	1
20	0.5	1	2
21	NA	0.5	1
22	0.5	0.5	1
23	0.5	0.5	≤ 0.5
24	0.5	1	1
25	2	2	1
26	0.5	1	≤ 0.5
27	0.5	0.5	1
28	0.5	0.5	1
29	0.5	0.5	1
30	0.5	1	1
31	?	0.5	2
32	>8	>8	1
33	>8	>8	4
34	0.25	0.5	1
35	0.25	0.5	1
36	0.25	0.5	2
37	0.25	0.25	1
38	0.25	0.25	≤ 0.5
39	0.25	0.5	1
40	0.25	0.5	1
41	0.25	0.5	1
42	0.25	0.25	1
43	0.25	1	1
44	0.25	0.5	1
45	0.25	0.5	1
46	0.25	0.25	1
47	0.25	0.25	1
48	0.25	0.5	1
49	0.25	0.25	1
50	0.25	0.5	1
51	0.25	0.25	4
52	0.25	0.25	4
53	0.06	0.5	1
54	0.06	0.5	≤ 0.5
55	0.125	0.5	≤ 0.5
56	0.125	0.25	≤ 0.5
57	0.125	0.25	≤ 0.5

Table 4. Sensitivity, specificity and validity of MBT ASTRA in comparison to CLSI results for caspofungin.

	CLSI versus ASTRA		
	Sensitivity	**Specificity**	**Validity**
C. albicans	100%	100%	88%
C. glabrata	94.2%	80%	95%

Discussion

MALDI-TOF MS has become a broadly applied rapid technology in clinical laboratories to identify yeasts and yeast-like isolates. Rapidness and accuracy of this method predestines it to be used in AFST. While standard AFST by microdilution methods takes at least 24 hours, applying MALDI-TOF MS may accelerate susceptibility testing. So far, rapid susceptibility testing by MALDI-TOF-MS has mainly been described for bacteria (23, 27, 30–33). One research group has reported about a rapid AFST approach by analysing similarities of MALDI-TOF MS profile spectra derived from different setups containing increasing antibiotic concentrations (34–36). MBT ASTRA is a semi-quantitative method that has first been applied for the detection of meropenem susceptibility in bacteria derived from culture plates or from positive blood cultures within 1 to 4 h (32). Subsequently, additional studies were performed demonstrating the successful applicability of MBT ASTRA for detection of resistant strains to gentamicin, cefotaxime (37) and rifampicin (31, 38). In all these studies the time to result was significantly reduced by the MALDI-TOF MS approach from at least 24h to 3-6 h. In this study, MBT ASTRA was optimized to facilitate the detection of resistance against caspofungin in two different yeast species, *C. albicans* and *C. glabrata*.

Since yeasts are growing significantly slower than bacteria, a major improvement of MBT ASTRA was the adaptation of the incubation time.

The usually required incubation time of 24 h up to even 72 h for yeast resistance detection by microdilution was reduced to a short incubation time of about 6h. To achieve MBT ASTRA results, the concentration of the internal standard was adapted to the profile spectra derived from yeasts that revealed different intensities and numbers of peaks than spectra derived from bacteria.

MBT ASTRA is a phenotypic assay directly monitoring the growth in the presence of antifungals and comparing this to the growth of a control setup without antifungal. In contrast, the approach of De Carolis *et al*. (34) analysed changes in the MS profile spectra induced by antifungals. A recent study of the same group has shown that this approach did not accurately work for *C. glabrata* strains resistant to anidulafungin with known *FKS2* mutations (36). MBT ASTRA has been set up based on microdilution methods that are generally accepted as the gold standard. A threshold for the relative growth at 0.6 was defined as cut-off value for categorization. RG values similar or below 0.6 were considered as susceptible and RG value above 0.6 were categorized as resistant. The breakpoint concentrations categorizing into susceptible and resistant strains defined for the MBT ASTRA were close to the breakpoint concentrations defined by CLSI and varied in this study by double concentration. For MBT ASTRA, breakpoints were defined as followed: S ≤ 1 µg/mL and S ≤ 0.5 µg/ml caspofungin and R > 1µg/mL and R > 0.5 µg/ml caspofungin for *C. albicans* and *C. glabrata*, respectively. Employing MBT ASTRA, no intermediate range could be defined.

Performing the MBT ASTRA on a serial dilution of a certain antifungal, an individual, assay-specific breakpoint of each strain was determined, and it was possible to categorize strains as susceptible, or resistant by reading the respective “MBT ASTRA MIC” which is double concentration higher. On the other hand, MBT ASTRA can be performed for a single antifungal concentration corresponding to the breakpoint of the antifungal to be tested. Such an experiment facilitates the simple and rapid categorization into susceptible and resistant strains. Compared to the established routine methods for AFST (24, 25, 39), the time to result of MBT

ASTRA is about seven hours, thus providing the resistance status at the same day. Even automated commercially available systems like Vitek 2 (BioMérieux, France) or Sensititre Yeast One (Thermo Fischer Scientific, USA) are significantly slower (17–19, 40). Molecular approaches that are generally faster must deal with the major obstacle that they do not provide any information about the phenotype of the respective strain. Additionally, altered gene sequences or new resistance mechanisms may not be detected by PCR analysis.

In this study, absolute concordance to the microdilution results was found for *C. albicans*. For seven slow growing strains insufficient growth of the control setups was observed preventing a reliable classification. This demonstrated the importance to check first the growth in the control setups. To realize this, a threshold for the minimum required growth calculated by the prototype software was introduced. Only strains with sufficient growth of the control were considered for evaluation of sensitivity and specificity of MBT ASTRA. Seven slow growing strains were detected that could not be evaluated resulting in an assay validity of 88%. These strains were also tested by VITEK 2 to find out whether they belong to other species, like *C. africana* or *C. stellatoidea,* which could not be detected by MALDI. The results confirmed that all seven strains were *C. albicans* and susceptible to echinocandins. Therefore, the poor growth rate cannot be explained by this. Additionally, caspofungin is known to show a high variability in the MIC distributions in different labs causing in wrong reporting of WT strains as non-susceptible for most common *Candida* species (41). This could explain the few cases of disagreement found in this study between MBT-ASTRA and CLSI. Beyond the growth rate, technical errors, like insufficient extraction, use of inadequate culture medium as well as inaccurate amount of internal standard, could lead to poor spectra quality bearing the risk of mis-classification. Besides, the reproducibility of this study revealed acceptable results for *C. albicans* with only 1 out of 30 analyses being incorrect. The detailed analysis of the spectra of the respective strain revealed a general problem with the MALDI-TOF MS measurements on

that day for that strain because spectra were only acquired from one spot instead of two spots. The visual inspection of these spectra revealed poor spectra quality. This could be a reason for the misclassification. Further improvements of the software algorithm will be necessary to evaluate the spectra quality and to exclude those spectra with insufficient quality.

For *C. glabrata*, sensitivity, specificity and validity were calculated to be 94%, 80% and 95%, respectively. Four strains were found susceptible against caspofungin by MBT ASTRA compared to 5 strains categorized as susceptible by microdilution. The relatively low specificity found in this study could be explained by the low number of only 5 susceptible *C. glabrata* strains included. One out of these 5 susceptible strains was categorized as resistant by MBT ASTRA. In contrast, two of 33 resistant strains were incorrectly detected as susceptible. The reproducibility study for *C. glabrata* revealed one deviation out of 30 analyses. One susceptible and two intermediate strains were mis-categorized. Furthermore, in this study MBT ASTRA could not identify intermediate strains. For *C. albicans,* no intermediates were tested and for *C. glabrata* except of one strain all intermediates were categorized as resistant. This might be further improved by modifications in the assay setup in the future.

Moreover, one susceptible and one resistant strain of *C. albicans* indicated an increase of growth at a caspofungin concentration of 4 µg/ml. Probably the same paradoxical effect that has been described for microdilution occurred also for MBT ASTRA (42) (Supplementary Figure 1 A, F). In principal, this approach has the potential to be applicable for susceptibility testing of further yeast species and different antifungals. Further investigations, possibly additional optimization, and validation will be necessary to develop the approach for routine application workflows.

In summary, this study provided promising results for AFST in *C. albicans* and *C. glabrata* by MBT ASTRA. Considering hands-on-time, assay setup is in principle identical to conventional microdilution methods. However, sample preparation requires some extra steps. After

incubation, additional work including centrifugation, extraction and spotting lysate on the target is necessary and takes about 15 min per strain. Considering that the resistance result is available on the same day, these efforts seem to be justified. In summary, the excellent outcome with respect to sensitivity and specificity, the simple setup, and the short incubation time predestinate this MALDI-TOF MS based approach as an interesting alternative for conventional AFST. Since MALDI-TOF MS instruments are already part of many clinical laboratories, no additional investment (excluding a centrifuge to deal with microtiter plates which is mostly available in the clinical labs) will be required to perform this cost-efficient and fast method in the future.

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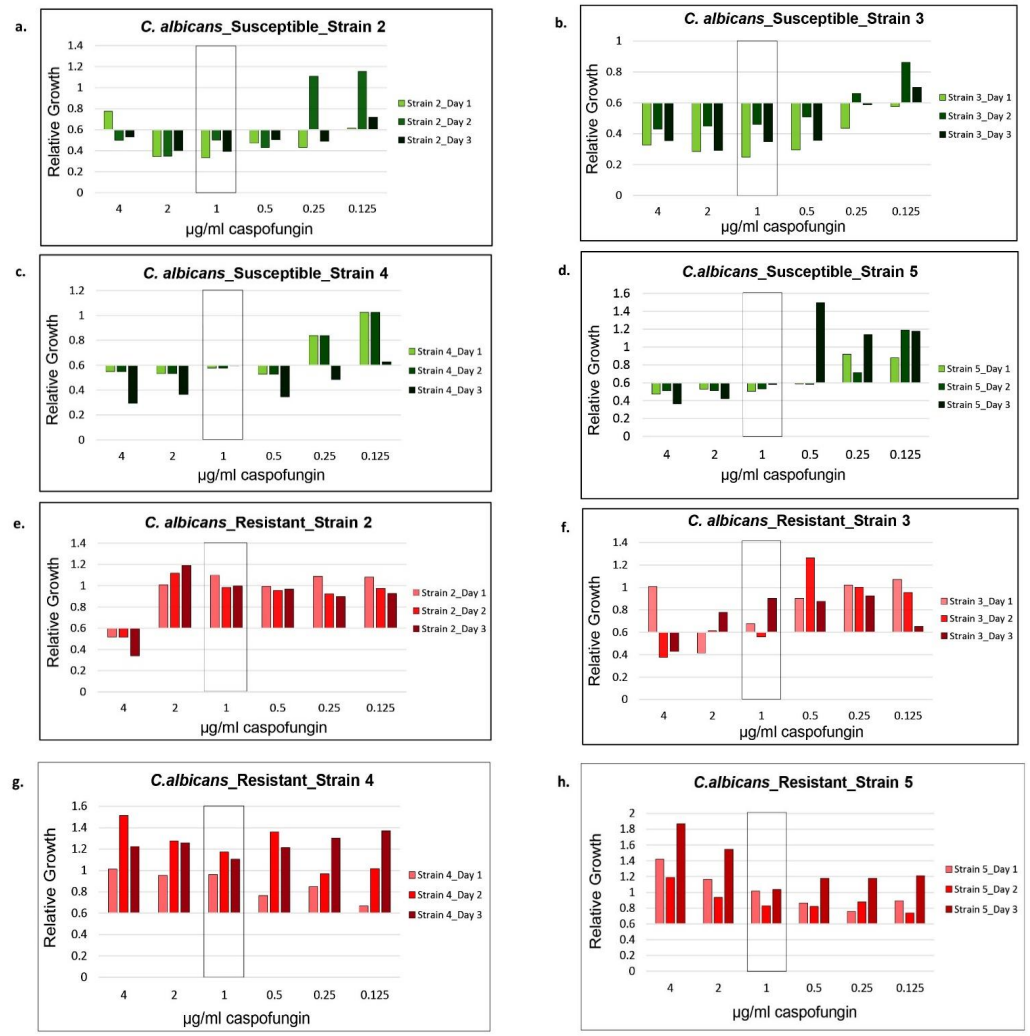
Supplementary Figure 1. Reproducibility of the MBT ASTRA. Relative growth values were obtained by two-fold serial dilutions of caspofungin for *Candida albicans* (susceptible (a-d), resistant (e-h)) and *Candida glabrata* (susceptible (i-l), resistant (m-p), and intermediate (q-s)) on three different days. *C. albicans* and *C. glabrata* revealed constant results at the breakpoint concentration (boxes) for the different experiments.



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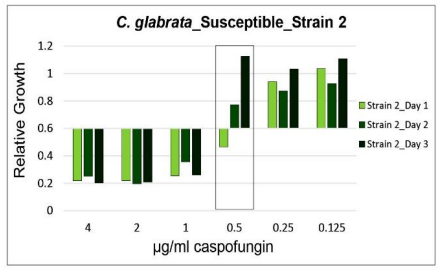

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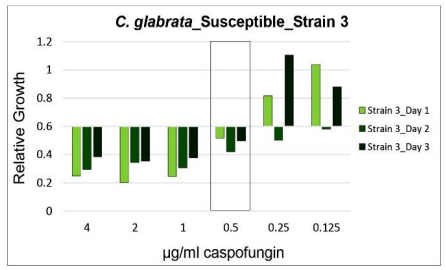

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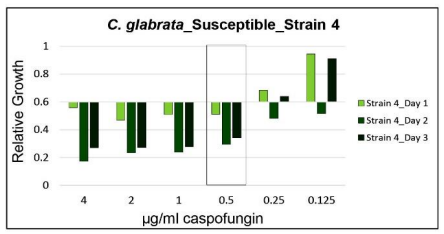

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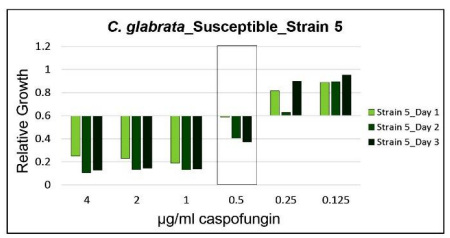

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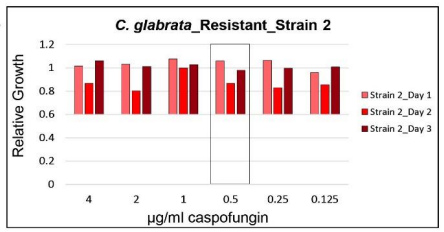

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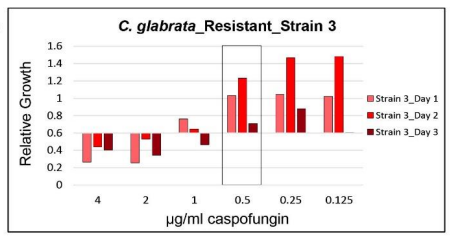

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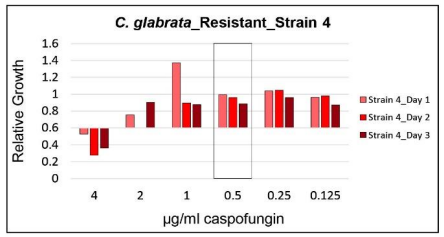

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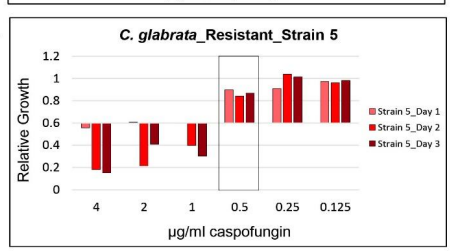

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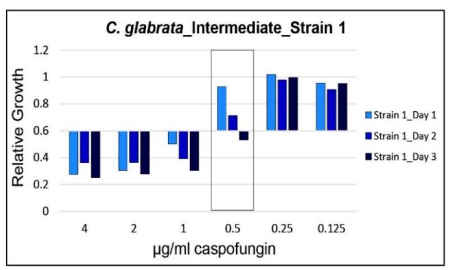

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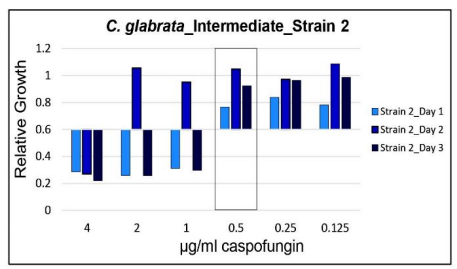

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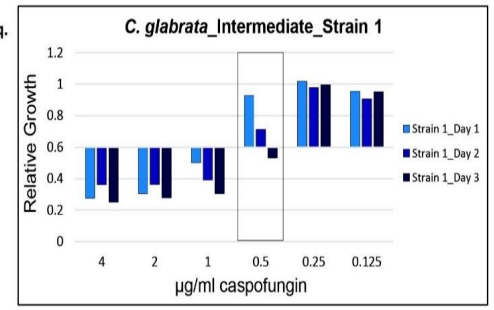

Chapter 4

Anidulafungin susceptibility testing of *Candida glabrata* isolates from blood cultures by the MALDI Biotyper Antibiotic (Antifungal) Susceptibility Test Rapid Assay

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Abstract

Echinocandins are the recommended first-line antifungals for treatment of invasive candidiasis. The increasing number of *Candida glabrata* strains resistant against echinocandins is an emerging health care concern. The rapid detection of resistant *C. glabrata* isolates is an urgent requirement for clinical laboratories. In this study, we developed the MALDI Biotyper antibiotic (antifungal) susceptibility test rapid assay (MBT ASTRA) for the rapid detection of anidulafungin-resistant *C. glabrata* isolates directly from positive blood cultures. Of 100 *C. glabrata* strains, MBT ASTRA classified 69 as susceptible and 29 as resistant. Microdilution assays performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines, used as a standard reference, identified 65 susceptible, 9 intermediate, and 26 resistant isolates. Sequencing of hot spot 1 and hot spot 2 regions of the *FKS1* and *FKS2* genes classified 86 susceptible and 14 resistant isolates. The MBT ASTRA had sensitivity and specificity of 80% and 95%, respectively, compared to the microdilution method. Positive and negative agreement of MBT ASTRA was calculated at 100% and 80%, respectively, compared with the molecular sequencing approach. Together, these results revealed a high accuracy of MBT ASTRA compared to microdilution according to the CLSI and PCR analysis, resulting in a categorical agreement of 90% and 83%, respectively. The validity of MBT ASTRA was 98%. Importantly, MBT ASTRA provided antifungal susceptibility testing (AFST) within 6 h that was both accurate and reliable compared to the other two approaches, which require at least 24 h or are costly. Therefore, this method has the potential to facilitate clinical AFST rapidly at low sample costs for clinical labs already equipped with matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS).

Keywords: *Candida glabrata*; MBT ASTRA; anidulafungin; positive blood samples; rapid antifungal susceptibility testing

Introduction

Invasive candidiasis is becoming more prevalent due to a significant increase in the number of high-risk patients (1). The epidemiology of candidiasis has changed over the last decades, and non-*albicans Candida* species make up a larger proportion of the *Candida*-related infections (1, 2). *Candida glabrata* is commonly reported as a major non-*albicans Candida* species involved in invasive candidiasis, with a prevalence of up to 30% (2–4). The increasing incidence of *C. glabrata* and the availability of only four antifungals for systemic treatment have a major impact on human health management. This is largely due to the widespread use of fluconazole, the most widely used antifungal, to which *C. glabrata* has acquired resistance. In addition, resistance to echinocandins, a first line therapy for most *Candida* species, appeared first in *C. glabrata* isolates (5–7). Echinocandins inhibit β-1,3 glucan synthase, which is encoded by *FKS1* and *FKS2* genes in *C. glabrata* (8–10). Resistance against echinocandins is due to mutations in the hotspot 1 and hotspot 2 regions (HS1 and HS2) of the *C. glabrata FKS1* and/or *FKS2* genes in *C. glabrata* (6, 11, 12).

Two conventional methods used in clinical laboratories to analyse antifungal susceptibility are microdilution assays performed according to the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). These methods detect resistant isolates using phenotypic analysis based on microbial growth properties for at least 24h, and up to 48h for isolating measures of antifungal tolerance (13). These long incubation times are a major drawback, and more rapid approaches are required (10, 14). Molecular techniques provide faster approaches to detect resistant yeast isolates. However, they do not provide phenotypic information and only report on known mutations. New resistant mechanisms will escape detection with these approaches (15, 16). Additionally, performing antifungal susceptibility testing (AFST) by PCR is costly per individual sample. To fulfil the advanced requirements for AFST, in particular for patients with invasive

candidiasis, clinical laboratories require rapid and accurate methods to distinguish *Candida* species and to detect isolates that are resistant to specific antifungals.

MALDI Biotyper antibiotic susceptibility test rapid assay (MBT ASTRA) was recently described as a potential rapid tool for antifungal susceptibility testing within 6 hours (10, 17). MBT ASTRA is a phenotypic assay that compares cell growth in the presence of an antifungal to the growth in a control setup without antifungal. This semi-quantitative MALDI-TOF MS based assay separates susceptible and resistant strains based on the acquired profile spectra (10). Thus far, this method has been optimized for detection of *C. albicans* and *C. glabrata* resistant against caspofungin derived from Sabouraud Dextrose Agar (SDA) (10), and for *C. auris* resistant against echinocandins derived from SDA and positive blood culture (17). Due to the rising incidence of isolates of *C. glabrata* strains resistant to echinocandins, here we evaluated the applicability of MBT ASTRA to *C. glabrata* strains resistant against anidulafungin directly derived from positive blood culture. MBT ASTRA results were compared to CLSI standard microdilution method and genotypic analysis of HS1 and HS2 of *FKS1* and *FKS2* genes.

Materials and methods

Strains. A panel of 100 *C. glabrata* isolates derived from either strain collections (19 isolates from the Westerdijk Institute, The Netherlands) or stock cultures of routine samples (81 isolates) was analyzed. The isolates were collected from different geographical distributions such as Austria, Israel, Germany, the Netherlands, the United States and derived from different human's samples including blood, urine and superficial wounds. All isolates were identified by MALDI Biotyper (Bruker Daltonik GmbH, Germany) and molecular analysis (PCR) (17–19). The isolates were stored at -80 °C and fresh overnight cultures on Sabouraud dextrose agar (SDA) were used for *in vitro* antifungal susceptibility testing assays by CLSI method and DNA

sequencing. Blood cultures were spiked with cells derived from fresh SDA cultures and used for *in vitro* antifungal susceptibility testing by MBT ASTRA. The reference strains *C. parapsilosis* ATCC 22019 and *Pichia kudriavzevii* (= *C. krusei*) ATCC 6258 recommended by CLSI for susceptibility testing were used as quality control strains (20). The reference strains *C. albicans* ATCC 64548 and *C. albicans* ATCC 64550 recommended as quality control strains by EUCAST (21) were applied for microdilution as well as for MBT ASTRA.

AFST by CLSI reference method. *In vitro* antifungal susceptibility testing was performed by microdilution method according to CLSI guideline M60 (November 2017) using twofold serial dilutions of anidulafungin (Pfizer, New York, USA) ranging between 0.06 and 8 µg/ml and a control without antifungal (20).

DNA extraction, PCR and sequencing. Genomic DNA was extracted using the TaKara kit (Takara, city, Japan) according to the manufacturer's recommendations (22). PCR oligonucleotide primers used for HS1 and HS2 of *FKS1* and *FKS2* genes were designed by Geneious program 11.0.4 and are described in Table 1. HS1 and HS2 of *FKS1* and *FKS2* were amplified by polymerase chain reaction (PCR) in a 50 µl volume containing 5 µl of buffer, 2 mM magnesium chloride, 0.25 µl of Taq polymerase enzyme (BIO-21040, BioLine Company, London, United Kingdom), 10 mM dNTP (BIO-39043, BioLine Company), 2 µl of template DNA, and 1 µl of individual primers (F and R). PCR was performed for 5 min pre-denaturation at 94 °C followed by 35 cycles including denaturation at 95 °C for 30 s, annealing at 58 °C for HS1 of *FKS1* and *FKS2* or 61°C for HS2 of *FKS1* and *FKS2* for 30 s, respectively, and subsequent extension at 72 °C for HS1 of *FKS1* and HS2 of *FKS1* and *FKS2* for 30 seconds

and for HS1 of *FKS2 for* 1min, respectively. Final extension was performed for 8 min at 72 °C.

PCR products were purified and subsequently directly sequenced by Sanger sequencing method in a total volume of 10 µl mixture including 0.5 µl of Big dye, 3 µl of green buffer, 2 µl of PCR products and 0.5 µl of each primer in separate tubes (23). The results were mapped to *C. glabrata* CBS 138 (http://www.candidagenome.org) as the reference.

MBT ASTRA on positive blood cultures. *In vitro* antifungal susceptibility testing against anidulafungin by MBT ASTRA was performed directly from positive blood culture as recently described (10, 17). Briefly, blood culture bottles (BD Bactec Plus Aerobic/F; Becton Dickinson, Heidelberg, Germany) were enriched with 10 ml whole sheep blood, subsequently spiked with the respective *C. glabrata* isolate, and incubated in a Bactec automated blood culture instrument (Becton Dickinson) until they were flagged positive. *C. glabrata* cells were isolated by MALDI Sepsityper kit (Bruker Daltonik GmbH, Bremen, Germany) according to the manufacturer's recommendations (17), and the pellet was re-suspended in 1 ml RPMI 1640 medium to prepare a suspension of McF 0.5. Twofold serial dilutions of anidulafungin ranging from 0.06 to 4 µg/ml and a control without antifungal were prepared. Subsequent steps of MBT ASTRA were performed as previously described (10, 17).

Data analysis. MBT ASTRA prototype software written in the freely available software package "R" was used to analyze the acquired spectra as described previously (10, 17, 24, 25). This prototype software individually calculated the area under the curve (AUC) for each setup, followed by comparison of the AUC for each antifungal concentration with the AUC of the control setup resulting in the relative growth (RG) that was calculated by the following equation: $RG = (\text{AUC}_{\text{RPMI+ anidulafungin}}) / (\text{AUC}_{\text{RPMI}})$.

The relative growth cut-off was set to 0.5 RG units for strains derived from positive blood cultures. Isolates showing RGs above this threshold were considered as resistant and strains with an RG below this cutoff value were considered as susceptible against anidulafungin. SeqMan Pro and Mega 7.0 programs were applied to analyze data acquired by gene sequencing. Venn diagram was created via the online program (http://bioinformatics.psb.ugent.be/webtools/Venn/). CLSI microdilution method and PCR were independently considered as standard methods. Sensitivity, specificity, very major error, major error and categorical agreement of MBT ASTRA were calculated by comparison with the CLSI method.

Results

Antifungal susceptibility testing by standard microdilution. CLSI microdilution method starting with colonies grown on solid agar medium was considered as the phenotypic gold standard for evaluating data acquired by MBT ASTRA and PCR. This method was used to differentiate susceptible and resistant strains after 24h (up to 48h for some slow-growing strains, and/or isolates tolerant to anidulafungin), respectively. The results were interpreted according to the breakpoints recommended by CLSI as follows: S ≤ 0.125 µg/ml, I = 0.25 µg/ml, and R ≥ 0.5 µg/ml. Most isolates wwere susceptible (n= 65) followed by resistant isolates (n= 26) and intermediate ones (n= 9) (Table 2).

Identification of mutations in *FKS1* and *FKS2* genes of *C. glabrata*. All isolates were screened for mutations in *FKS1* HS1, HS2, and *FKS2* HS1, HS2 after growing on SDA medium. In total, 14 of 100 isolates had mutations in HS1 of either *FKS1* or *FKS2*; no mutations were detected in HS2 in either of these genes. Eighty-six isolates had no mutations and were

considered susceptible (Table 2). All 14 isolates mutated in *FKS1* and *FKS2* genes showed high MIC values against anidulafungin determined by CLSI microdilution (Supplementary Table S1). All detected mutations in *FKS1* and *FKS2* HS1 are summarized in Table 1.

Table 1. Gene sequencing of hot spot regions 1 (HS1) and 2 (HS2) of *FKS1* and *FKS2* of *Candida glabrata*. Mutations found in HS1 of *FKS1* and *FKS2* were described and labelled in bold red color. No mutations were detected in Hotspot 2 of *FKS1* and *FKS2*.

Locus	Hotspots	Primers	Primer sequence	Mutations (FLILSLRDP)
***FKS1* HS 1**	TTC TTG ATT CTA TCT CTA AGA GAT CCA	*FKS1* HS1F	5´-AGTCTACCAGACGTTACGTC- 3´	**4 Isolates** SLILSLRDP, FLILPLRDP, FLIPSLRDP, FLILSLRGP
***FKS1* HS 1**	TTC TTG ATT CTA TCT CTA AGA GAT CCA	*FKS1* HS1R	5´-GAAAATGTTTCTCCATGGAGTC- 3´	
***FKS1* HS 2**	GAC TGG GTC AGA CGT TAC ACA TTA	*FKS1* HS1F	5´-CTA GGT ACA CAA CTT CCA ATT GA- 3´	No mutations found
***FKS1* HS 2**	GAC TGG GTC AGA CGT TAC ACA TTA	*FKS1* HS1R	5´-AAT CGC TCA ACA AAG CAG AT- 3´	
***FKS2* HS 1**	TTC TTG ATT TTG TCT CTA AGA GAC CCT	*FKS2* HS1F	5´-GGTATCCTAGGTGTTAATCTT- 3´	**11 Isolates** FLILPLRDP, SLILSLRDP, VLILSLRDP, FLILSLRGP, FLILFLRDP, FLILSLREP, FLILSLRDT
***FKS2* HS 1**	TTC TTG ATT TTG TCT CTA AGA GAC CCT	*FKS2* HS1R	5´-CAGTTTCTGAACATGATCGATA- 3´	
FKS2 HS 2	GAT TGG ATC AGA CGT TAT ACA TTG	*FKS2* HS1F	5´-TAG GTA CAC AAT TGC CCG TA- 3´	No mutations found
***FKS2* HS 2**	GAT TGG ATC AGA CGT TAT ACA TTG	*FKS2* HS1R	5´-TGT CAC TCA ATA GAG CAG CA- 3´	

Antifungal susceptibility testing by MBT ASTRA. Analysis of 100 *C. glabrata* isolates derived directly from positive blood cultures (without the requirement for growth on SDA medium, taking approximately 27 hours vs 67 hours to obtain results by CLSI method) revealed that a relative growth cut-off value of 0.5 yielded the best agreement with the microdilution results. Based on this, MBT ASTRA breakpoints for anidulafungin were defined as follows: susceptible ≤ 0.5 µg/ml and resistant ≥ 1 µg/ml. MBT ASTRA could not detect intermediate isolates. Therefore, isolates were classified either as susceptible or resistant strains. Two susceptible strains detected within 48h by CLSI method, did not pass the growth control cutoff

and were excluded from the data analysis. Therefore, validity of MBT ASTRA was calculated 98%. Accordingly, MBT ASTRA categorized 69 isolates as susceptible and 29 isolates as resistant against anidulafungin within 6h (Table2).

Table 2. *In vitro* anidulafungin susceptibility testing using CLSI microdilution, MBT ASTRA and molecular analysis for 100 *Candida glabrata* isolates. Detection of susceptible, resistant and intermediate strains by 3 different methods are shown.

Methods		
CLSI	**PCR**	**MBT ASTRA**
Total No (Susceptible/ Intermediate/ Resistant)	Total No (Susceptible/ Resistant)	Total No (Susceptible/Resistant)
100 (65/ 9/ 26)	**100** (86/14)	**100** (69/29)

Comparison between microdilution, PCR and MBT ASTRA for detection of resistant and susceptible *C. glabrata* isolates. Table 3 A, B and figure 1 summarize the comparison of the three methods for susceptibility/resistance of *C. glabrata* against anidulafungin, respectively. PCR of HS1 and HS2 was used to detect resistant strains and 14 isolates were determined to carry respective mutations in HS1 of *FKS1* and *FKS2* genes. All these isolates also were identified as resistant by CLSI microdilution method and MBT ASTRA, yielding a sensitivity (positive percent agreement) of 100% for these two approaches (Figure 1, Table 3). According to the number of susceptible isolates that were identically detected by PCR, the CLSI method and MBT ASTRA, specificity (negative percent agreement) and categorical agreement of MBT ASTRA and CLSI microdilution methods compared to PCR were calculated as 80%, 83%, and 75%, 79%, respectively (Table 3). In contrast, compared to the microdilution, only 14 of the 26 isolates detected as resistant by CLSI method were classified as resistant by PCR, which

reduced PCR sensitivity to 54%. Twelve resistant isolates and nine intermediate isolates detected by CLSI method were susceptible by PCR resulting a specificity of 100% for PCR. Categorical agreement of PCR compared to microdilution method was calculated 79% (Table 3).

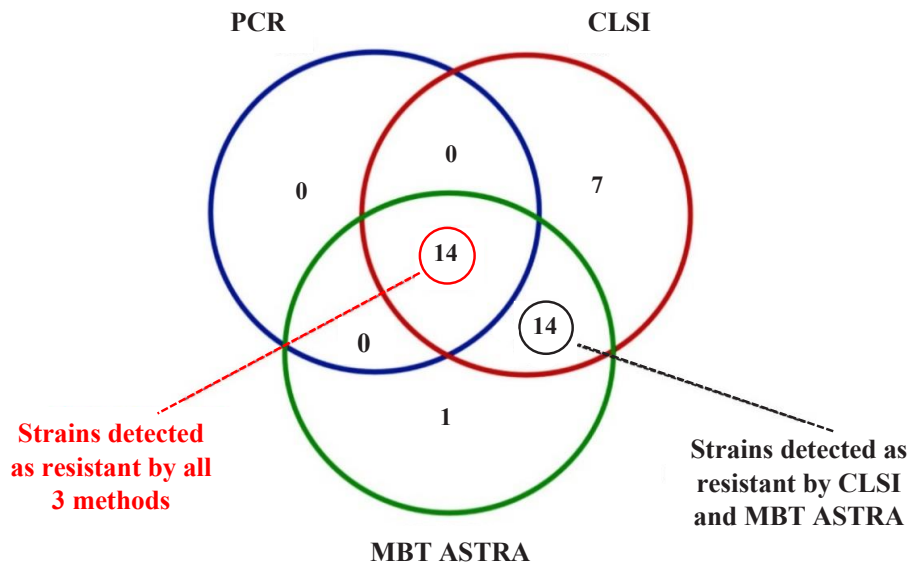

Figure 1. Venn diagram of 35 *Candida glabrata* isolates found resistant by PCR, the CLSI method, and the MBT ASTRA for AFST. The number of resistant strains detected by each method is shown. In total, 14 isolates resistant against anidulafungin were detected by all three methods. There were no isolates detected as resistant only by PCR. Fourteen resistant isolates were detected by CLSI microdilution and the MBT ASTRA but not by PCR. The CLSI method detected 7 resistant isolates (6 intermediate and 1 resistant) that were found to be susceptible by PCR and MBT ASTRA. Three isolates were detected as susceptible by PCR and the CLSI method but not by MBT ASTRA. However, 2 out of these 3 isolates could not pass the growth control cutoff and were not considered for data analysis.

MBT ASTRA AFST results were obtained more rapidly than PCR, being performed directly on positive blood culture vs MIC microdilution tested on SDA (27 hours vs 67 hours). In total, 25 *C. glabrata* isolates were classified as resistant by MBT ASTRA and CLSI methods. MBT

ASTRA detected one further isolate as resistant that had been classified as susceptible by CLSI microdilution. Conversely, one isolate was detected as resistant by CLSI microdilution that was classified as susceptible by MBT ASTRA. By CLSI microdilution, nine isolates were detected as intermediate. Including the intermediate category into the resistant category resulted in a misclassification of 6 isolates by MBT ASTRA. Detection of intermediate isolates has not yet optimized by MBT ASTRA; therefore, minor error could not be calculated (Figure 1, 2). Sensitivity, specificity and categorical agreement of MBT ASTRA compared to the microdilution method, considering intermediate isolates as resistant isolates, were calculated as 80%, 95%, and 90%, respectively (Table 3).

Table 3. Sensitivity, specificity and categorical agreement of MBT ASTRA compared to CLSI microdilution and PCR method.

	CLSI microdilution		PCR	
	MBT ASTRA	**PCR**	**MBT ASTRA**	**CLSI**
Sensitivity	80%	40%	100%	100%
Specificity	95%	100%	80%	75%
Categorical agreement	90%	79%	83%	79%

Further data analysis was performed, and very major error and major error were calculated with 4% and 1% for MBT ASTRA compared to the CLSI method, respectively. Figure 2 provides an example of the RG and MIC distribution of 28 *C. glabrata* (14 resistant and 14 susceptible) that were correctly detected by all three methods. Additionally, Figure 2 shows disagreeing results obtained by CLSI and MBT ASTRA methods for 8 isolates.

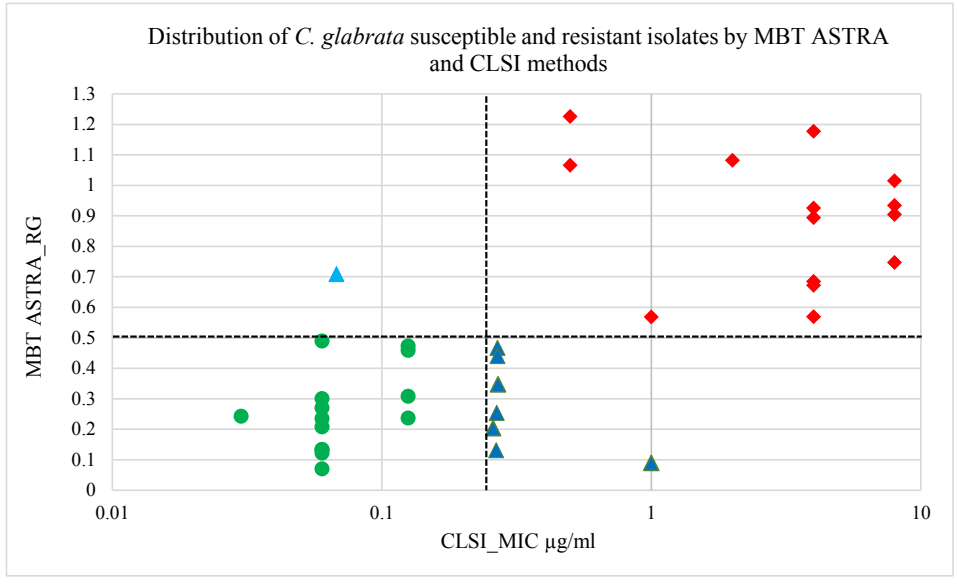

Figure 2. Example of RG and MIC distribution of 36 *Candida glabrata* isolates against anidulafungin derived from positive blood cultures. RG values (y-axis) of 36 susceptible and resistant isolates were plotted against the MIC values (x-axis). The horizontal dashed line indicates the suggested cutoff for MBT ASTRA derived RG values. The vertical dashed line indicates the MIC breakpoint defined by CLSI. Fourteen susceptible isolates (green dots) and 14 resistant isolates (red squares) were separated similarly by MBT ASTRA and CLSI method. Eight isolates with discrepancy of the results between these two methods were shown in triangle shapes. One isolate was detected as resistant by MBT ASTRA (light blue triangle) that was susceptible by CLSI method. In opposite, CLSI microdilution detected 6 intermediate and 1 resistant *C. glabrata* isolates (dark blue triangle) that all were susceptible by MBT ASTRA.

Discussion

MBT ASTRA already was optimized for *C. glabrata* and *C. albicans* species derived from SDA against caspofungin within 7 hours (10). However, *C. glabrata* bloodstream infections have been increasing in prevalence (2). Therefore, we evaluated MBT ASTRA for its applicability to susceptibility testing of positive blood cultures against anidulafungin, the echinocandin widely used in clinical laboratories for reliable echinocandin susceptibility

testing. In the current study, the comparison of MBT ASTRA on positive blood culture with PCR and CLSI microdilution from overnight cultivation on SDA revealed high sensitivity. MBT ASTRA results from blood culture were identical to those obtained by CLSI microdilution method tested from SDA cultures.

Importantly, MBT ASTRA provided results directly from positive blood cultures on the same day, i.e. 48 to 72 hours earlier than did the CSLI microdilution. Therefore, MBT ASTRA is a rapid method based on growth in the presence vs absence of antifungal. This difference is critical, as rapid diagnosis is required for the fast implementation of optimal therapeutic strategies.

Compared to the CLSI method breakpoints for *C. glabrata* against anidulafungin by MBT ASTRA had to be increased by two serial dilution steps to S ≤ 0.5 µg/ml and R ≥ 1 µg/ml to achieve concordance to the CLSI method. MBT ASTRA is a novel approach developed for a short incubation time of 6h incubation. It seems that an increased antifungal concentration is required to get a separation within this short time frame. The only drawback of MBT ASTRA is the disability of this method to detect intermediate isolates. It caused misclassification of six out of nine intermediate isolates as susceptible by MBT ASTRA causing a reduction of sensitivity to 80%. While excluding intermediate isolates from resistant category causes an increase of the sensitivity of MBT ASTRA by 96%. In a recent study performed by the same group, MBT ASTRA was already set up for *C. glabrata* isolates derived from SDA against caspofungin applying a relative cut-off value of 0.6 RG unit, and 18 out of 19 *C. glabrata* isolates classified as intermediate by the CLSI method were correctly detected as resistant by MBT ASTRA (10). Misclassification of intermediate isolates as susceptible may lead to therapeutic failure and even might cause the induction of new strains resistant to the respective antifungal. Furthermore, the 2 isolates detected as resistant by MBT ASTRA but as susceptible by microdilution method and PCR showed very poor spectra in the respective setup. Only

strains with sufficient growth in the control setups were considered for further evaluation of sensitivity and specificity of the MBT ASTRA method. Thus, two isolates that could not pass the growth control cutoff were not included in the data analysis. This is in agreement with our earlier observations that slow-growing or antifungal-tolerated *C. glabrata* and *C. albicans* isolates susceptible to caspofungin (10) could not be correctly classified because a certain degree of growth is necessary for AFST evaluation. MBT ASTRA and CLSI methods are both based on the growth of microorganisms and are phenotypic assays, therefore enough growth is also crucial to evaluate AFST by CLSI and EUCAST microdilution methods. Since MALDI Sepsityper kit was used to isolate *C. glabrata* cells from blood samples, it could happen that the lysis buffer treatment may have a negative effect on the cell growth, resulting in the acquisition of poor spectra. Increasing cell inoculation might overcome this issue. However, concentration of antifungal would have been calibrated based on an increasing cell inoculation, and this would require a new set up in future studies. On the other hand, six hours incubation time may not provide an appropriate growth rate for all *C. glabrata* isolates and even for other yeast species in the future. This difficulty might be resolved by adding extra supplements into the medium to accelerate cell growth. In addition, further optimization of the workflow could improve this evaluation.

Although CLSI microdilution method is considered as the phenotypic gold standard in this study, it is not the method always used for susceptibility testing in clinical laboratories. Other methods frequently used include E-test strips that are not suitable for resistance testing of all *Candida* species against antifungals due to trailing growth of some species at the inhibition zone (26). Here, we found that MBT ASTRA identified more susceptible strains than did the CLSI method. High specificity of MBT ASTRA prevents mistreatment of patients that also may cause an upcoming resistance to the other drugs. Given that some isolates considered susceptible by CSLI microdilution remained difficult to clear (27, 28), comparison of the

clinical outcomes for antifungal responses of the relevant strains with conflicting classification would help to determine which assay is clinically more relevant. Calculated very major error and major error between these two methods was about 4% and 1%, respectively, which indicates the potential of MBT ASTRA to be applied for rapid AFST on patient blood culture samples in the near future in order to improve therapy management.

In general, the MIC breakpoints introduced by CLSI for different yeast species against different antifungals are based on pharmacokinetic/pharmacodynamic data, outcome data, MIC distribution, and the presence or absence of mutations in the isolates (6, 29). According to the gene mutations, only 14 out of 100 isolates had resistance-related mutations in HS1 of *FKS1* and *FKS2* genes; all these isolates showed elevated MIC values by both CLSI and MBT ASTRA methods. Eleven of these had mutations in HS1 of *FKS2* and 4 isolates had HS1 mutations in *FKS1;* 1 isolate had mutations in HS1 of both genes. The different HS1 mutations did not result in different of MIC values. This contrasts with previous studies that suggested that *C. glabrata* isolates harbouring D666Y mutation in *FKS2* confer weak resistance while S663P mutation confer strong resistance (30, 31). Additionally, we did not find any mutations in HS2 and like previous studies, most of the mutations influencing *C. glabrata* echinocandin susceptibility were located in HS1 of either *FKS* gene (6, 8, 32). We sequenced only the HS1 and HS2 regions of *FKS1* and *FKS2* genes, and thus could have missed any mutations in other regions of these genes or in other genes. This could explain the lower number of resistant isolates determined by PCR versus those detected by MBT ASTRA and microdilution. Of course, whole genome sequencing can provide an extensive assessment of genetic mutations involved in antifungal resistance (33, 34), but it is not yet a practical solution for AFST, as it is time-consuming and relatively costly. In another study, AFST based MALDI- TOF assay was developed to detect *C. glabrata* isolates resistant to anidulafungin within 3h. This method was based on the detection of changes in the protein profile spectrum relative to the antifungal

concentration by MALDI-TOF MS. AFST-based MALDI- TOF assay was useful to detect strains with mutations in *FKS1*, however, it failed to detect *C. glabrata* resistant isolates carrying known *FKS2* mutations (35).

Fourteen resistant isolates were detected by MBT ASTRA that had no HS mutations in *FKS* genes. In the routine clinical practice where usually no gene sequencing is performed, it has obviously not been noticed that there are *C. glabrata* isolates with elevated MIC values for echinocandins without mutations in *FKS1* and/or *FKS2* genes (30, 36). However, there are some possible explanations for this observation, which is also known as adaptive response (37, 38). For instance, upon extensive exposure to different antifungals, *C. glabrata* activates stress responses to compensate for the growth inhibition and to avoid death. Accordingly, growth of *C. glabrate* isolates on plates containing even low concentrations of echinocandins can cause the emergence of resistant isolates (30). Furthermore, exposure to echinocandins activates the cell wall integrity (CWI) pathway in *C. glabrata*. The CWI regulates glucan synthesis via *RHO1* activation and cell wall repair. Rho1 triggers activation of protein kinase C (PKC1) and upregulation of the *FKS* genes. Both events increase *C. glabrata* resistance to echinocandins (37, 39). Activation of CWI also is linked to other cell wall components like chitin. More chitin affects the cell wall permeability, leading to elevated MIC values *in vitro* (38, 39). Such adaptive responses can be induced by environmental stresses, temperature, ionic, oxidative, and osmolarity changes, and thereby result in reduced susceptibility of *C. glabrata* to different antifungals (39).

In summary, MBT ASTRA provides a rapid method for testing of *C. glabrata* antifungal susceptibility against echinocandins with cells directly obtained from positive blood cultures, with results available within 7 hours. These results support those of earlier studies (10, 17). Further studies focusing on other *Candida* species and antifungals, as well as tests using clinical isolates, rather than type strains used here, will need to be performed to validate the clinical

applicability of the approach. In particular, the detection of strains classified as intermediate by CLSI requires further improvement. Therefore, further optimization of MBT ASTRA for blood culture derived cells is required prior to introduction of the method in the routine AFST. Nonetheless, MBT ASTRA provides a promising AFST approach to obtaining rapid results with high accuracy compared to the molecular and standard microdilution methods. The setup of this approach is simple, cost-effective, and once a MALDI-TOF MS instrument is already available in the clinical laboratory for identification, no additional investment, lab space and training of lab stuff is required. Along with all the benefits of MBT ASTRA, further improvement will be necessary to get a fully automated method. Taken together, this study and previously studies employing MALDI-TOF MS based susceptibility approaches have significant implication on the multifunctional use of MALDI-TOF MS instruments in the next future. This instrument may not only be used for species identification but can also concurrently be employed for AFST and AST.

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Conflict of interest

M.K., K.S., and M.V. are employees of the mass spectrometry company Bruker Daltonik GmbH.

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Supplementary Material Table 1. Overview about the 100 *Candida glabrata* strains used in this study. Results of in vitro anidulafungin susceptibility testing using CLSI and MBT ASTRA methods are shown. Detected mutations at HSP1 and HSP2 of *FKS1* and *FKS2* genes are displayed. Strains detected as resistant are highlighted in light grey and those detected as intermediate isolates according to CLSI microdilution results are highlighted in dark grey.

Strains	CLSI (MICs)	MBT ASTRA (MICs)	*FKS1*				*FKS2*			
			Hot spot 1	Mutations	Hot spot 2	Mutations	Hot spot 1	Mutations	Hot spot 2	Mutations
1	0.125	0.25	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
2	0.06	0.125	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
3	0.06	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
4	0.06	0.125	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
5	0.06	0.25	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
6	0.06	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
7	0.125	0.06	TTCTTGATTCTATCTTTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
8	1	0.125	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
9	1	4	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
10	4	4	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGCCTCTAAGAGACCCT	FLILPLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
11	0.25	0.25	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
12	0.5	2	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TCCTTGATTTTGTCTCTAAGAGACCCT	SLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
13	1	>4	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	GTCTTGATTTTGTCTCTAAGAGACCCT	VLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
14	1	2	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
15	2	>4	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
16	0.06	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
17	0.125	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
18	0.125	0.25	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
19	0.25	>4	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
20	0.125	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
21	2	>4	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
22	0.125	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
23	0.125	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
24	0.125	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
25	0.06	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
26	0.06	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
27	0.06	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
28	0.06	>4	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
29	0.06	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
30	0.06	0.125	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
31	0.06	0.125	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
32	0.06	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
33	0.25	>4	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
34	0.06	0.25	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
35	0.06	0.25	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
36	0.125	0.25	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
37	0.06	0.25	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
38	0.125	0.5	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
39	0.125	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
40	>8	>4	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGCCTCTAAGAGACCCT	FLILPLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
41	0.06	0.125	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
42	0.06	0.25	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
43	0.06	0.125	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
44	0.06	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
45	0.06	0.25	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
46	0.06	0.25	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
47	0.06	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
48	0.06	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
49	0.06	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
50	0.03	0.125	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
51	0.06	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
52	0.25	>4	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
53	0.25	0.5	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
54	0.06	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
55	0.06	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
56	0.06	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
57	0.125	0.125	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
58	0.25	0.125	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
59	0.06	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
60	0.06	NA	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
61	0.06	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
62	0.06	0.125	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
63	0.125	0.25	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
64	0.125	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
65	0.125	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
66	0.125	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
67	0.06	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
68	0.06	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
69	1	>4	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
70	0.5	>4	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
71	0.06	NA	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
72	0.125	0.125	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
73	0.06	0.125	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
74	0.06	0.125	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
75	0.25	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
76	0.125	0.25	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
77	0.125	0.25	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
78	1	>4	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
79	0.25	0.125	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
80	0.06	0.125	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
81	0.5	>4	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
82	0.5	>4	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
83	0.06	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
84	0.06	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
85	0.25	0.06	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
86	0.06	0.125	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
87	0.5	>4	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
88	0.06	0.25	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
89	0.06	0.125	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
90	4	>4	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
91	0.5	>4	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TCCTTGATTTTGTCTCTAAGAGACCCT	SLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
92	4	>4	TCCTTGATTCTATCTCTAAGAGATCCA	SLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
93	4	>4	TTCTTGATTCTACCTCTAAGAGATCCA	FLILPLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
94	8	>4	TTCTTGATTCTATCTCTAAGAGGTCCA	FLILSLRGP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACCCT	FLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
95	2	>4	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	GTCTTGATTTTGTCTCTAAGAGACCCT	VLILSLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
96	4	2	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGCCTCTAAGAGACCCT	FLILPLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
97	8	>4	TTCTTGATTCCATCTCTAAGAGATCCA	FLIPSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGGCCCCT	FLILSLRGP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
98	4	>4	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTTCTAAGAGACCCT	FLILPLRDP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
99	4	>4	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGAACCT	FLILSLRELP	GATTGGATCAGACGTTATACATTG	DWIRRYTL
100	8	>4	TTCTTGATTCTATCTCTAAGAGATCCA	FLILSLRDP	GACTGGGTCAGACGTTACACATTA	DWVRRYTL	TTCTTGATTTTGTCTCTAAGAGACACCT	FLILSLRDPT	GATTGGATCAGACGTTATACATTG	DWIRRYTL

*NA (Not applicable): These strains were detected as resistant due to not passing the growth control cutoff. Therefore, respective isolate was deleted from data analysis

Chapter 5

Candida auris identification and rapid antifungal susceptibility testing against echinocandins by MALDI-TOF MS

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Abstract

Candida auris was first reported in an ear swab from Japan in 2009; it then promptly spread over five continents and turned into a global nosocomial problem. The main challenges faced by many researchers are the misidentification by conventional methods in clinical laboratories and failure in treatment. About 90% of *C. auris* strains are intrinsically resistant to fluconazole (FLU), and it is developing resistance to multiple classes of available antifungals. Echinocandins are the most potent class of antifungals against *C. auris*; however, reduced susceptibility to one or many echinocandin drugs has been recently observed. Thus, the main issues addressed in this paper are the fast and accurate identification of *C. auris* derived from Sabouraud dextrose agar and blood culture bottles as well as the rapid antifungal susceptibility test by MALDI-TOF MS. This study successfully identified all isolates of *C. auris* (n= 50) by MALDI-TOF MS, with an average log score of ≥ 2. An accuracy of 100% was found on both agar plate and blood culture bottles. MALDI Biotyper antibiotic susceptibility test-rapid assay (MBT ASTRA) was used for rapid antifungal susceptibility testing (AFST)**.** A comparison between MBT ASTRA and the Clinical and Laboratory Standards Institute guidelines (CLSI) detected a sensitivity and specificity of 100% and 98% for anidulafungin, and 100% and 95.5% for micafungin, respectively. A categorical agreement of 98% and 96% was calculated for the two methods. For caspofungin, sensitivity and specificity of 100% and 73% were found, respectively, with a categorical agreement of 82%. MBT ASTRA has the great potential to detect *C. auris* isolates non-susceptible against echinocandin antifungals within 6 h, which makes it a promising candidate for AFST in clinical laboratories in the future.

Keywords: *C. auris*, MALDI-TOF MS, rapid antifungal susceptibility testing, echinocandins

Introduction

Candida auris is a recently-emerged *Candida* species first isolated from human samples of the external ear in Japan in 2009 (1) that spread to more than 30 countries in less than 10 years (2). *C. auris* is primarily detected in patients with a long period of hospitalization in intensive care unit (ICU). It causes diseases ranging from superficial skin infections to invasive bloodstream infections (BSI) with high mortality rates (30% to 60%) (3–8). In some hospitals in Asia *C. auris* is the second most common isolated species from blood cultures (9). *C. auris* is resistant to fluconazole (FLU), and is also regularly reported as a multidrug resistant (MDR) yeast (6), (10), (11). Previous published studies showed increased *C. auris* MICs to all three major antifungal classes (6), (12–15). Echinocandins are the most effective drugs against C. auris infections (16), (17). The majority of studies have proposed a tentative MIC breakpoint (in µg/mL) for resistance i.e., ≥ 4 for anidulafungin and micafungin, and a MIC breakpoint ≥ 2 for caspofungin (18–20). In addition, echinocandin-resistant *C. auris* isolates have been observed on rare occasions in different geographical areas (15). Notably, 36% of echinocandin resistant *Candida* strains have cross-resistance to azole antifungals (14)**,** (15)**,** (21). Thus, knowing the degree of echinocandin resistance in specific *C. auris* isolates is critical for choosing appropriate antifungal drug therapeutic strategies**.** Considering the MDR propensity of this yeast the treatment of *C. auris* remains a challenge as also its identification in the routine microbiology laboratories (18), (20). *C. auris* has been misidentified as *Candida haemulonii*, *Debaryomyces hansenii* (= *C. famata*) and even *Saccharomyces cerevisiae* by conventional biochemical techniques and some commercial methods (18), (22), (23). Several studies have reported misidentification of *C. auris* as *C. haemulonii* by VITEK 2, if the database is not updated (20), (24), (25). Molecular methods remain the most reliable and accurate available approaches, although they are expensive and not used routinely in clinical laboratories (18–20). Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-

TOF MS) has recently been considered as a convenient, rapid and high throughput technology in the identification of variant microorganisms at the species level (26–28). Some studies showed that MALDI-TOF MS can identify *C. auris* correctly and rapidly, differentiating it from other related species (5), (29–31). However, the specificity and sensitivity of MALDI-TOF MS to a collection of *C. auris* isolates have not been tested. Taken together, identification and antifungal susceptibility tests for *C. auris* infection remain a challenge in the clinical microbiology setting.

Here, we investigated the MALDI-TOF MS based identification of *C. auris*. In addition, since a new trend to apply MALDI-TOF MS for AFST has appeared, Vella and collaborators have developed a rapid new AFST assay based on MALDI-TOF MS (44, 45). They analysed changes in the MS profile spectra induced by antifungals after 3h of incubation (44). Although this method successfully worked for *C. albicans* isolates resistant to caspofungin (44), it did not accurately work for *C. glabrata* strains resistant to anidulafungin with known *FKS2* mutations (45). Therefore, more recently, MALDI Biotyper antibiotic susceptibility test-rapid assay (MBT ASTRA) was introduced for the rapid detection of *C. albicans* and *C. glabrata* strains resistant against caspofungin (32), where resistant strains were detected within six hours with a high sensitivity and specificity for both species. This method is a semi-quantitative phenotypic assay based on the comparison of cell growth in the presence of an antifungal to growth in a control setup without antifungal drug. The present research explores, for the first time, the capability of the MBT ASTRA to detect *C. auris* strains non-susceptible against the echinocandin drug class, and the degree to which they agree with results from CLSI microdilution assays.

Materials and methods

***Candida auris* isolates.** A total of 50 *C. auris* isolates derived from either strain collections (10 from the Centers for Disease Control and Prevention, United States and 23 isolates from the Westerdijk Institute, The Netherlands) or stock cultures of routine samples (17 isolates) of different geographical areas (India, Israel, South Africa) were analysed (Supplementary Table S1). All isolates had already been identified as *C. auris* by different commercial and molecular techniques. The isolates were stored at -80°C and fresh overnight cultures on Sabouraud dextrose agar (SDA) were used for identification and antifungal susceptibility tests (AFST). The reference strains *C. parapsilosis* ATCC 22019 and *Pichia kudriavzevii* (= *C. krusei*) ATCC 6258, were used as quality control strains for susceptibility testing by the CLSI microdilution method. Two *C. auris* strains, CBS 12372 (KCTC 17809) and CBS 10913 (DSM 21092), were used as quality control strains for echinocandin susceptibility testing by the CLSI method and the MBT ASTRA (33), (34).

MALDI-TOF MS species identification. For the MALDI-TOF MS-based identification of *C. auris* the Bruker MBT Compass Library, Revision E MBT 7854 MSP Library was employed. Samples derived from agar plate were prepared according to the MALDI Biotyper standard protocol (35–37).

For species identification from blood cultures, blood culture bottles (BD Bactec Plus Aerobic/F; Becton Dickinson, Heidelberg, Germany) were enriched with 10 ml whole sheep blood and spiked with the respective *C. auris* strains. The bottles were incubated in a Bactec automated blood culture instrument (Becton Dickinson, Heidelberg, Germany) until they were positive for each isolate. Subsequently, the positive blood cultures were purified by MALDI Sepsityper kit (Bruker Daltonik GmbH, Germany) according to the manufacturer's recommendations (35), (36), (38).

CLSI microbroth susceptibility testing. The Minimum Inhibitory Concentration (MICs) for anidulafungin (Pfizer, New York, United States), micafungin (Astellas, Toyama, Japan) and caspofungin (Sigma-Aldrich, Germany) were determined in duplicate by the CLSI standard microdilution method according to guideline M60 (November 2017) (33). Slow-growing strains that could not be visually evaluated after 24 h, were analyzed after 48 h incubation at 37°C. So far, respective breakpoints for susceptibility/resistance classification have not been defined by CLSI and are suggested in this study.

MBT ASTRA. The *in vitro* antifungal susceptibility test was carried out according to the standard MBT ASTRA method, as recently described (32), using twofold serial dilutions of anidulafungin (ranging from 0.125 to 8 µg/ml), micafungin (ranging from 0.5 to 32 µg/ml), and caspofungin (ranging from 0.125 to 4 µg/ml) and an additional control without an antifungal. Briefly, supplemented RPMI 1640 medium (Sigma-Aldrich, Germany) and cell inoculum were prepared according to the EUCAST reference method (32), (34). Incubation was performed at 37°C in a Thermo Mixer (Eppendorf, Germany) under agitation at 300 rpm for 6h. Multi-well filter plates (1ml well, 0.45µm GHP, PALL, United States) were used to collect the cells after incubation by centrifugation at 4000×g for 5min (Eppendorf, Germany). Next, the cells were rinsed twice with 200 µl sterile deionized water and once with 100 µl 75% ethanol. Cell lysis was performed by 10.5 µl 70% formic acid (Merck, Germany) and 10.5 µl 100% acetonitrile (Roth, Germany) directly on the filter and was repeated once again. For MALDI-TOF MS measurements, 1 µl lysate of each set up was spotted in duplicate onto a polished steel target plate (Bruker Daltonik, Germany) and overlaid with 1 µl MALDI matrix (10 mg/ml of α -cyano-4-hydroxy-cinnamic acid [α-HCCA] in 50% acetonitrile–2.5% trifluoroacetic acid; Bruker Daltonik, Germany) containing the MBT ASTRA Standard II

(Bruker Daltonik, Germany). MALDI-TOF MS spectra were acquired and analyzed by MBT ASTRA prototype software (26), (27), (32).

MBT ASTRA from positive blood cultures. Twenty isolates from the strains described above were randomly selected, namely susceptible (n= 14) and non- susceptible (n= 6) strains to anidulafungin and micafungin, susceptible (n=13) or non- susceptible (n=7) to caspofungin. Preparation of blood cultures, incubation, and purification of positive cultures by MALDI Sepsityper kit was performed exactly as described above for strain identification. The pellet derived from the Sepsityper kit was resuspended in 1 ml RPMI 1640 medium and used to prepare cell suspension of McF 0.5. All following steps of MBT ASTRA were performed as described above (32).

Data analysis. MBT ASTRA prototype software written in "R" was employed for spectra analysis according to the procedure described by Lange *et al.* (32), (38), (39). First, the area under the curve (AUC) was calculated which is directly corresponding to the growth of the strain within the respective setup. Subsequently, the relative growth was calculated as a measure of the growth of the respective strain in the presence of antifungal: $RG = (AUC_{RPMI+antifungal})/(AUC_{RPMI})$. The relative growth cut-off was set to 0.7 RG units for all experiments. Strains with an RG value above this threshold were considered as non-susceptible; strain with an RG similar or below 0.7 were considered as susceptible. The CLSI microdilution was considered as standard method for evaluation of MBT ASTRA. Since this study was only a proof-of-principle-study, the number of tested strains was limited for applying a specific statistical calculation.

Results

Strain identification. All 50 strains of *C. auris* grown on SDA were correctly identified based on the MALDI Biotyper. The results, as shown in Table 1, indicate that 47 strains were identified with log (score) values of ≥ 2 and three strains with log (score) values ranging between 1.7-2. Likewise, identification from positive blood culture bottles detected 46 strains with high log (score) values ≥ 2 and only 4 strains with log (score) values ranging between 1.7-2. An absolute accuracy of 100% was calculated for *C. auris* identification using SDA and blood culture bottles (Table 1).

Table 1. Identification of 50 *Candida auris* isolates derived from SDA plate agar and positive blood cultures by MALDI-TOF MS, respectively.

Strains/ Culture medium	Number of strains	No of log (score) values			No of Correct Identified Samples (%)	Accuracy
		≤ 2	1.7-2	≥ 1.7		
C. auris / Plate agar (SDA)	50	47	3	-	50 (100%)	100%
C. auris / positive blood culture (BC)	50	46	4	-	50 (100%)	100%

CLSI antifungal susceptibility test. The CLSI microdilution method was considered as the gold standard to evaluate the MICs obtained by the MBT ASTRA. Since the CLSI has not yet determined the breakpoints for *C. auris* against echinocandins, the MICs found in this study based on CLSI microdilution method, were considered as a reference to evaluate breakpoints obtained by MBT ASTRA. Accordingly, strains were divided into 44 susceptible and 6 non-susceptible strains against anidulafungin and micafungin. All six isolates harbored an S639F mutation in FKS1 gene as published previously (15). Applying the CLSI method for caspofungin divided strains into 33 susceptible and 17 non-susceptible isolates (Table 2). All

6 non-susceptible strains confirmed by sequencing, were correctly detected by CLSI method. As shown in table 3, the MICs based on CLSI microdilution were defined as follows: R > 4 µg/ml and S ≤ 4 µg/ml for anidulafungin, R > 8 µg/ml and S ≤ 8 µg/ml for micafungin, and R ≥ 1 µg/ml and S < 1 µg/ml for caspofungin, respectively (Table 3) (Supplementary Table S1).

Table 2. *In vitro* echinocandin class drugs susceptibility test using CLSI microdilution and MBT ASTRA for 50 *Candida auris* isolates.

	Total No (Susceptible/Resistant)		
	Anidulafungin	**Micafungin**	**Caspofungin**
CLSI	50 (44/6)	50 (44/6)	50 (33/17)
MBT ASTRA	50 (43/7)	50 (42/8)	50 (24/26)

***In vitro* susceptibility test by MBT ASTRA.** MBT ASTRA was performed for 50 strains as described above, and the area under the curve (AUC) was calculated by MBT ASTRA prototype software, based on the acquired spectra according to the growth of each strain at each antifungal concentration (39). Further analysis determined that the minimal antifungal concentrations resulting in an RG value similar to or below the cut-off value of 0.7 were considered as the breakpoint for the respective strain. The RG values for four test strains (2 susceptible and 2 non-susceptible) are shown in figure 1.

For anidulafungin, the AUCs of the non-susceptible isolates were the same in absence and presence of the antifungal resulting in RGs close to 1; while for the susceptible strains, the AUCs were remarkably decreased in the presence of the drug resulting in decreasing RG values with increasing antifungal concentrations. The RG values of 42 susceptible strains already decreased at a concentration of ≤ 2 µg/ml of anidulafungin and for only one susceptible strain

at a concentration of 4 µg/ml. An example of the respective boxplot is given in Figure 1. Next, the MICs derived from MBT ASTRA were determined, and subsequently compared to the MICs derived from CLSI microdilution (Figure 2, Table 3). Accordingly, 4 µg/mL was defined as the anidulafungin breakpoint concentration. Findings indicated that 49 of 50 strains were classified correctly, with only one susceptible strain misclassified by MBT ASTRA.

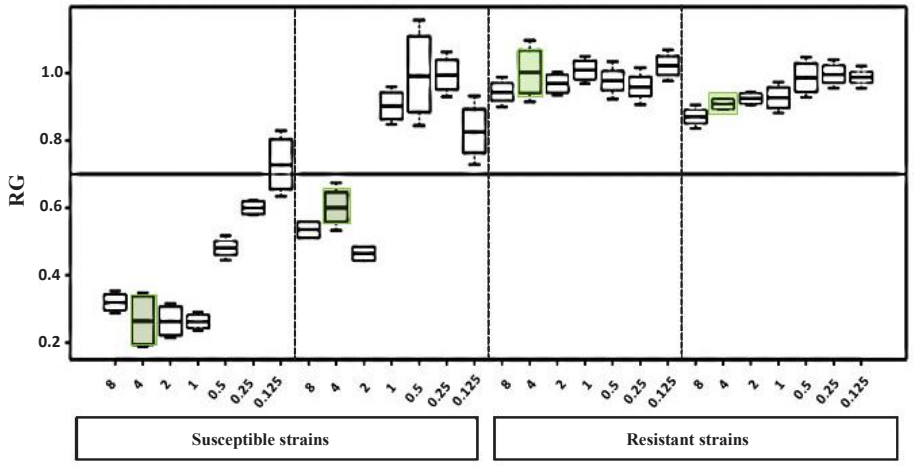

Figure 1. Results of the MBT ASTRA prototype software evaluation. The relative growth values of twofold serial dilutions of anidulafungin of 2 susceptible and 2 resistant *Candida auris* strains after 6 h incubation show distinct differences. The susceptibility/resistance threshold was set at a RG value of 0.7. For susceptible strains, at the concentration of 4 µg/ml anidulafungin a significant reduction of the relative growth was observed.

For micafungin, susceptible strains had a growth reduction only at higher concentrations of micafungin compared to anidulafungin. For micafungin at a concentration of 8 µg/mL, 42 susceptible strains were below the cut-off of 0.7. In contrast, all non-susceptible strains constantly grew above the cut-off of 0.7 for all concentrations up to 32 µg/ml. Table 3 shows

that micafungin MBT ASTRA-MICs were in high agreement with the MICs derived from CLSI microdilution. Accordingly, there is a clear separation of susceptible and non-susceptible strains for micafungin with a breakpoint concentration of 8 µg/ml for both the CLSI reference method and MBT ASTRA. In addition, analysis of caspofungin susceptibility of the isolates found that the RGs of susceptible isolates were decreased at concentrations of 2 µg/ml, while the growth for non-susceptible strains at this concentration was in the same range that had been observed for the controls for all caspofungin concentrations. The breakpoint for the MBT ASTRA was one serial dilution higher than the separating MIC for the CLSI method (Table 3).

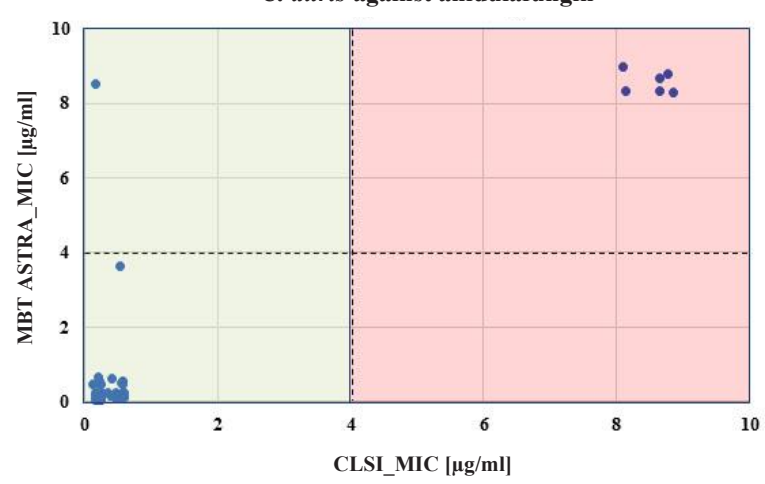

Figure 2. Comparison of CLSI microdilution MICs and MBT ASTRA MICs for *Candida auris* isolates (n= 50) against anidulafungin. For each isolate, the MIC value derived by MBT ASTRA (*y* axis) was plotted against the MIC obtained by microdilution (*x* axis) for anidulafungin (A). The colored boxes indicate the MIC ranges according to CLSI; green: susceptible and red: resistant. The horizontal dashed line indicates the suggested cutoff defined for MBT ASTRA MIC, and the vertical dashed line shows the suggested cutoff determined for CLSI. A high agreement was observed between both approaches, and only one isolate susceptible against anidulafungin was wrongly detected by MBT ASTRA.

Table 3. Comparison of suggested breakpoints against echinocandin class drugs for *Candida auris* tested by CLSI microdilution and MBT ASTRA. Susceptible and resistant are shown by S and R, respectively.

Methods	MIC µg/ml					
	Anidulafungin		Micafungin		Caspofungin	
CLSI	R> 4 µg/ml	S≤ 4 µg/ml	R> 8 µg/ml	S≤ 8 µg/ml	R≥ 1 µg/ml	S< 1 µg/ml
MBT ASTRA (SDA)	R> 4 µg/ml	S≤ 4 µg/ml	R> 8 µg/ml	S≤ 8 µg/ml	R> 2 µg/ml	S≤ 2 µg/ml
MBT ASTRA (blood culture bottles)	R> 1 µg/ml	S≤ 1 µg/ml	R> 4 µg/ml	S≤ 4 µg/ml	R≥ 1 µg/ml	S< 1 µg/ml

Overall, analysis of all 50 tested isolates by MBT ASTRA indicated a high agreement between anidulafungin and micafungin. One susceptible isolate was mislabeled by both antifungals, but all 6 strains determined as being non-susceptible by microdilution were correctly categorized by MBT ASTRA (Table 2). For anidulafungin, a high agreement of 98%, and sensitivity and specificity of 100% and 98% were calculated, respectively. For micafungin, categorical agreement was 96%, and sensitivity and specificity were 100% and 95.5%, respectively (Table 4). In contrast, for caspofungin twenty-four susceptible and twenty-six non-susceptible isolates were detected by MBT ASTRA (Table 2). All 17 strains classified as non-susceptible by the CLSI method were determined also as non-susceptible by MBT ASTRA. However, 9 out of 33 strains that were categorized as susceptible by microdilution, were detected as non-susceptible by MBT ASTRA. A categorical agreement of 82% was obtained between the two methods, and sensitivity and specificity of 100% and 73% were calculated, respectively (Table 4, Supplementary Table S1).

Table 4. Sensitivity, specificity and categorical agreement of MBT ASTRA in comparison to CLSI microdilution results for anidulafungin, micafungin and caspofungin.

MBT ASTRA	Anidulafungin	Micafungin	Caspofungin
Sensitivity	100%	100%	100%
Specificity	98%	95.5%	73%
Categorical agreement	98%	96%	82%

MBT ASTRA on positive blood culture. The applicability of the MBT ASTRA for detecting yeast strains non-susceptible to echinocandins was directly investigated for cells derived from seeded blood cultures. Initially, spectra were analyzed by the MBT ASTRA prototype to determine the breakpoints and thresholds for the respective three antifungals. Afterwards, 14 susceptible and 6 non-susceptible strains (against anidulafungin and micafungin) were tested at the respective breakpoints and thresholds. The results for anidulafungin showed that the breakpoint was decreased to 1 µg/mL at a threshold of 0.7, compared to the results of cells cultured on SDA (Table 3). Beside this result, the breakpoint was reduced to 4 µg/mL for micafungin when an RG threshold of 0.7 was applied (Table 3). All 6 non-susceptible and 14 susceptible strains were correctly separated at these breakpoints of the corresponding antifungals and thresholds (Figure 3). For caspofungin, 6 out of 7 non-susceptible strains and twelve out of thirteen susceptible strains were correctly detected. In this case, the 2 strains that were misidentified, had been correctly detected by MBT ASTRA from colonies grown on SDA. These data demonstrate that the breakpoint for caspofungin must be decreased to 1 µg/mL at a threshold of 0.7 (Table 3) when using MBT ASTRA.

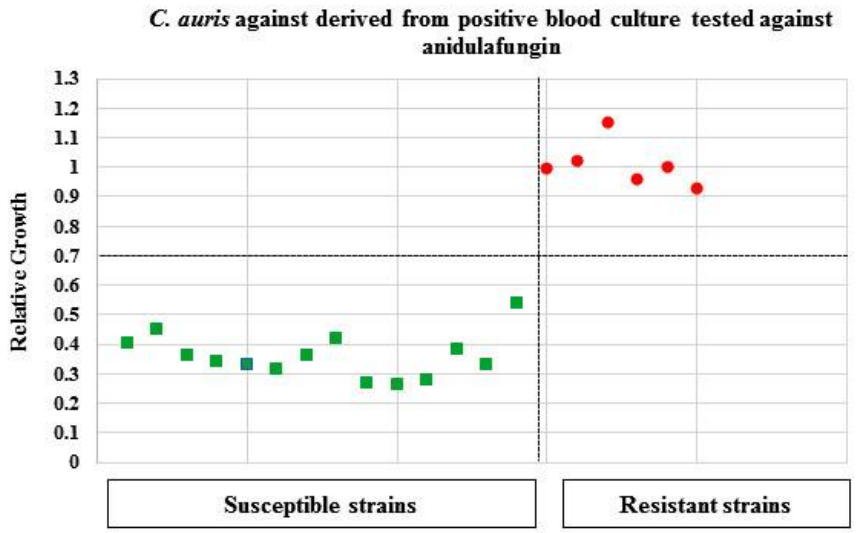

Figure 3. MBT ASTRA of *Candida auris* isolates derived from positive blood cultures. A total of 20 *C. auris* isolates (resistant= 6, susceptible= 14) derived from positive blood cultures were tested against anidulafungin. The MBT ASTRA breakpoint was determined at 1 µg/ml and an RG threshold of 0.7 for anidulafungin (A). All resistant and susceptible isolates were correctly detected.

Discussion

This study investigated the identification of 50 *C. auris* isolates by MALDI-TOF MS, suggested CLSI microdilution cut-offs and evaluated MBT ASTRA for AFST on echinocandin. The results from this study demonstrate that a rapid MALDI-TOF MS technology not only can be used to unequivocally identify *C. auris*, but it has the potential to be successfully applied in rapid AFST. Recently, attention has focused on the identification of *C. auris* to distinguish it from close relatives like *C. haemulonii* using diverse biochemical-based testing, molecular and commercial methods. A total of 50 *C. auris* isolates were correctly identified by MALDI-TOF MS with an accuracy of 100% when cultured on SDA and isolated

from positive blood cultures. This was for a first time that applicability of MALDI-TOF MS was used to identify *C. auris* derived from positive blood cultures. This finding broadly supports the work of other studies in this area, demonstrating the high accuracy of MALDI-TOF MS for the identification of *C. auris* based on a well-established reference database, compared to other available methods. For instance, the results of this study are in agreement with those obtained by Bao and collaborators , who more recently showed a high performance of the MALDI-TOF MS in identifying 23 clinical isolates of *C. auris* that were cultured on three different media Their study indicated that the highest log scores were achieved for isolates grown on SDA (40).

Additionally, the present study demonstrates the successful applicability of MBT ASTRA for the detection of *C. auris* isolates non-susceptible to echinocandin drugs cultured on SDA. The most interesting finding was that 49 and 48 out of 50 *C. auris* isolates were correctly labeled as either susceptible or non-susceptible to anidulafungin and micafungin by MBT ASTRA, respectively. A sensitivity of 100% indicated that all six isolates non-susceptible against the respective antifungals were successfully detected. Notably, the same susceptible isolate was misidentified as resistant for both antifungals, even after repetition. The reason for this observation could be that this strain revealed an MIC in the microdilution that is next to the breakpoint concentration. Considering that this strain had not been sequenced together with the allowed variance of the microdilution, it could be that this strain is indeed resistant. Further analysis is required to clarify this result.

To achieve valid results by MBT ASTRA, it is essential to first check the growth in the control setups. This was done by introducing a threshold for the minimum required growth calculated by the prototype software. Those strains with sufficient growth in the control setups were considered for further evaluation of sensitivity and specificity of the MBT ASTRA. Taken together, categorical agreement between MBT ASTRA and the CLSI was very high, namely

98% and 96% for anidulafungin and micafungin, respectively. Moreover, in the present study, the cut-offs found by the CLSI reference method for *C. auris* anidulafungin susceptibility testing confirmed the results obtained by other studies (15, 18, 21, 41), and the breakpoint found by MBT ASTRA was identical to the suggested CLSI method by the Centers for Disease Control and Prevention . Importantly, the same breakpoint was obtained much more rapidly by MBT ASTRA, which requires up to 6h, compared to 24to 48h used for the CLSI method. For micafungin, the suggested cut-offs for the CLSI microdilution and MBT ASTRA were also identical, but these cut-offs were higher compared to those of other studies (15, 21, 41). A recent study reported that micafungin to be the most potent echinocandin with an MIC approximately equal to that of anidulafungin (41). In this study, the higher MICs obtained by the CLSI method and MBT ASTRA might be an epidemiological impact on fungal infections with a shift towards a reduced susceptibility of the strains against echinocandins (13), (14). Notably, results with caspofungin had lower specificity and sensitivity than anidulafungin or micafungin, with a categorical agreement between the CLSI microdilution and MBT ASTRA of 82%. Furthermore, the breakpoint found by MBT ASTRA was one serial dilution higher than that applied for the CLSI method. In accordance with these results, a previous study reported challenges in identification of *FKS1* WT *C. auris* isolates for caspofungin antifungal susceptibility testing due to the presence of an 'Eagle effect' (42). This phenomenon has been notably reported for the caspofungin-caused reduction of the antifungal activity at high concentrations (42). As mentioned in the study by Espinel-Ingroff *et al.,* variability of caspofungin MICs has been observed for *Candida* species using CLSI and EUCAST methods from different clinical laboratories. They reported that most of the wild-type (WT) isolates (e.g., *C. glabrata* and *C. krusei*) were detected as either non-WT or resistant isolates (43). Accordingly, Kordalewska *et al.* (2018) examined AFST of caspofungin for 106 *C. auris* isolates and found that only isolates with mutations in *FKS1* were resistant to echinocandins.

Therefore, it is recommended that routine microdilution methods be avoided when detecting resistant strains without gene sequencing (41). However, the reduced susceptibility of *C. auris* isolates to one or more echinocandin class drugs has been reported in several studies (21, 41). A further extension of this MBT ASTRA investigation was to analyse its performance on positive blood culture samples. For anidulafungin and micafungin, all susceptible and non-susceptible strains were labelled correctly, with complete agreement between the CLSI method and MBT ASTRA. The breakpoint concentration was decreased by two and one serial dilution(s) for anidulafungin and micafungin, respectively. For caspofungin, one susceptible and one non-susceptible strain were *mis*-labelled resulting in a categorical agreement of 90%. Thus, for caspofungin, these results from blood culture bottles were more in agreement with CLSI method than those obtained from SDA.

Findings reported here shed new light on rapid and accurate antifungal susceptibility testing for echinocandin by MALDI-TOF MS. A new performance of MALDI-TOF MS for rapid AFST based on changes in the profile spectra in the presence of antifungals was recently introduced by Vella *et al.* (44), but they revealed that this approach could not be perfectly applied for *C. glabrata* strains resistant to anidulafungin with known *FKS2* mutations (45). In this study, the CLSI method was considered as the gold standard, however, it is not a perfect method and some susceptible isolates are not clearly detected by the CLSI method. Hence, the MBT ASTRA might be even more helpful in the future. In summary, MBT ASTRA has been shown to be applicable for rapid AFST in *C. albicans*, *C. glabrata* (32), and *C. auris*. Applicability for further yeast species and antifungals will be necessary.

Conflict of Interest: M.K., K.S., and M.V. are employees of the mass spectrometry company Bruker Daltonik GmbH.

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Ethical Approval: According to common sense, for this study no ethical approval from an ethics committee is required, because all strains were derived either from strain collections or stock cultures of routine samples. The isolates used in this non-clinical in vitro study were obtained during routine patient care for which no written informed consent is required. The local Institutional Review Board of the hospital determined that ethics approval and consent from admitted patients was not required according to national and institutional guidelines.

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Chapter 6

Evaluation of Microsatellite Typing, ITS Sequencing, AFLP Fingerprinting, MALDI-TOF MS, and Fourier-Transform Infrared Spectroscopy Analysis of *Candida auris*

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Abstract

Candida auris is an emerging opportunistic yeast species causing nosocomial outbreaks at a global scale. A few studies have focused on the *C. auris* genotypic structure. Here, we compared five epidemiological typing tools using a set of 96 *C. auris* isolates from 14 geographical areas. Isolates were analyzed by microsatellite typing, ITS sequencing, amplified fragment length polymorphism (AFLP) fingerprint analysis, matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS), and Fourier-transform infrared (FTIR) spectroscopy methods. Microsatellite typing grouped the isolates into four main clusters, corresponding to the four known clades in concordance with whole genome sequencing studies. The other investigated typing tools showed poor performance compared with microsatellite typing. A comparison between the five methods showed the highest agreement between microsatellite typing and ITS sequencing with 45% similarity, followed by microsatellite typing and the FTIR method with 33% similarity. The lowest agreement was observed between FTIR spectroscopy, MALDI-TOF MS, and ITS sequencing. This study indicates that microsatellite typing is the tool of choice for *C. auris* outbreak investigations. Additionally, FTIR spectroscopy requires further optimization and evaluation before it can be used as an epidemiological typing method, comparable with microsatellite typing, as a rapid method for tracing nosocomial fungal outbreaks.

Keywords: *Candida auris*; molecular epidemiology; epidemiological typing; nosocomial outbreak

Introduction

Candida auris was described in 2009 in Japan and since then it has caused infections at a global scale with a serious nosocomial health risk (1–3). In less than a decade, *C. auris* was isolated on all six human-inhabited continents (4, 5), and infections were reported from more than 40 countries (4, 6, 7). Nosocomial *C. auris* outbreaks were first reported from South Korea (8), followed by India (9, 10), South Africa (11), Kuwait (12), Venezuela (13), USA (14), and European countries (2, 3, 7). The phylogeographic structure of *C. auris* has been studied using whole genome sequencing (WGS) and internal transcribed spacer (ITS) sequencing. These studies suggested that the *C. auris* isolates belong to four separate geographic clades, namely Eastern Asia (Korea and Japan), Southern Asia (India and Pakistan), South Africa, and South America (Venezuela). Importantly, a fifth lineage was detected recently from a patient in Iran that never traveled outside the country. This Iranian isolate possessed >200,000 single-nucleotide polymorphisms (SNPs) relative to isolates from the other four clades and, thus, may represent a fifth clade (15, 16). Isolates belonging to the four currently recognized clades differ from each other by tens to hundreds of thousands of SNPs, and exhibit limited within-clade diversity of only tens of base pairs (1, 17). However, the isolates within one clade with limited genomic diversity may have broad physiological variety (17, 18). Therefore, using different genotyping and biochemical techniques could present a clear overview of the geographical distribution of typing *C. auris* isolates. A few studies have investigated the geographical distribution of *C. auris* by other genotyping techniques, such as amplified fragment length polymorphism (AFLP) fingerprinting (2, 13, 18) and microsatellite typing (19). The latter was used for earlier population structure analysis of several pathogenic *Candida* species (19–22). ITS sequencing was used not only for the identification of *Candida* isolates, but also for typing purposes (17, 18, 23, 24). Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) is widely used for the rapid and accurate identification of

yeasts, including *C. auris* (18, 25–30), and is also applied to *C. auris* strain typing (18, 27, 31). The IR Biotyper (Bruker Daltonics, Bremen, Germany) was recently introduced into the field of microbial strain typing. This rapid and straightforward system is based on Fourier-transform infrared (FTIR) spectroscopy and uses molecular vibration fingerprints, primarily of the C-O stretching of biomacromolecules, e.g., carbohydrates, to characterize a microbial sample by strain-specific absorbance patterns in the infrared spectrum (32). The IR Biotyper was recently developed for clinical applications, such as the study of nosocomial outbreaks and their dynamics, in order to prevent the spread of pathogens inside the hospitals.

FTIR spectroscopy had been applied to type isolates of *Saccharomyces cerevisiae* and was able to differentiate between different *Saccharomyces* species (33), but also for the analysis of cell wall structures (34, 35). The application of this method to type bacterial species in hospital outbreaks was investigated several times. For instance, typing clinical *Klebsiella* isolates (32, 36) and Gram-negative bacilli clones was successfully performed using FTIR spectroscopy (IR Biotyper) (37). A few studies have examined the role of FTIR spectroscopy in the typing of clinical isolates (38). In this study, we compared different typing techniques, namely microsatellite typing, AFLP fingerprinting, ITS sequencing, MALDI-TOF MS, and IR Biotyper FTIR spectroscopy, to evaluate their application in typing *C. auris* isolates to further contribute to their epidemiology and dispersal routes in order to improve hospital hygiene and patient management.

Materials and methods

Candida auris isolates and media

Isolates used in this study were collected from different geographical regions, and belonged to the previously defined four major clades based on molecular studies (1, 14, 17, 19, 26, 39). A set of 96 *C. auris* isolates was obtained from the Westerdijk Fungal Biodiversity Institute,

Utrecht, The Netherlands (*n* = 37), the Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA; *n* = 10), and individual researchers (*n* = 49). The majority of isolates came from India (*n* = 38) (2, 9, 10), followed by 58 isolates from other geographical areas, namely Austria (from a Turkish patient) (*n* = 1) (40), Belgium (from a Kuwaiti patient) (*n* = 2) (41), Japan (*n* = 3) (42), Israel (*n* = 9) (43, 44), Korea (*n* = 3) (8), Malaysia (*n* = 3) (45), Oman (*n* = 13) (46, 47), Pakistan (*n* = 2), Saudi Arabia (*n* = 2) (48), Spain (*n* = 13) (49), South Africa (*n* = 4), Switzerland (*n* = 1) (50), and Venezuela (*n* = 2) (13) (Supplementary Table S1). The isolates were identified by MALDI-TOF MS (Bruker Daltonik) using routine settings (27, 51), and the Bruker MBT Compass Library, Revision E (7854 reference entries). The isolates were stored at −80 ˚C and cultured onto Sabouraud dextrose agar (SDA) overnight at 37 ˚C before further analysis.

Microsatellite typing

All 96 strains were subjected to a recently developed 12-loci microsatellite typing scheme (19). Genomic DNA of all *C. auris* isolates was extracted by the CTAB method as described elsewhere (52). The twelve loci were amplified using monoplex PCR, and forward primers were all fluorescently labelled with fluorescein for subsequent detection by capillary electrophoresis on an ABI3730xL Genetic Analyzer platform (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA). Raw data were processed using BioNumerics v7.6 (Applied Maths, Sint-Martens-Latem, Belgium) and a UPGMA dendrogram was generated with BioloMICS v12 (BioAware, Hannut, Belgium).

Internal transcribed spacer region (ITS) sequencing

The amplification of the internal transcribed spacer (ITS) region was performed using primers ITS1 and ITS4, followed by Sanger sequencing using the same primers, as described elsewhere

(44). Raw sequence data were checked and contigs were compiled with SeqMan v12 (DNASTAR, Madison, WI, USA). BioloMICS v12 (BioAware) was used to analyze sequence data and create a UPGMA dendrogram. ITS sequences were deposited in GenBank under accession numbers MN242989–MN243084.

Amplified fragment length polymorphism (AFLP) fingerprinting

The genetic diversity of the *C. auris* isolates was assessed by AFLP fingerprinting as described elsewhere (53). The selective primers used in the current study were HpyCH4IV with one selective residue (5`-FLU-GTAGACTGCGTACCCGTC-3`) and the MseI primer with four selective residues (5`-GATGAGTCCTGACTAATGAT-3`). PCR products were purified and diluted 10× in ddH_2O. One microliter of diluted PCR product was added to a mixture of 8.9 µL ddH_2O and 0.1 µL Orange 600 internal size marker (Nimagen, Nijmegen, The Netherlands) followed by 1 min heating at 100 °C, and thereafter directly analyzed on an ABI3730xL Genetic Analyzer (Applied Biosystems). Raw data were imported into BioNumerics v7.6 (Applied Maths) and automatically processed. The assignment of the internal size standard was visually checked and manually corrected if needed. A UPGMA dendrogram was generated with BioloMICS v12 (BioAware).

MALDI-TOF mass spectrum analysis

Overnight cultured *C.auris* isolates grown on SDA were used for spectra acquisition. Full extraction was performed according to the MALDI Biotyper standard protocol as described elsewhere (54–56). Briefly, from each isolate, biomass was taken with a 10 µL inoculation loop and suspended in 500 µL distilled water and centrifuged 3 min at 14,000× *g*, the supernatant was discarded, and 1 mL of 70% ethanol was added. The suspension was homogenized and then centrifuged at 3 min at 14,000× *g*. Following this, based on the pellet

size, equal amounts of 70% formic acid and 100% acetonitrile were added. After centrifugation for 5 min at 14,000× *g*, 1 µL of supernatant was spotted eight times onto a polished steel target plate (Bruker Daltonik) and overlaid with 1 µL MALDI matrix (10 mg/mL of α-cyano-4-hydroxy-cinnamic acid (α-HCCA) in 50% acetonitrile–2.5% trifluoroacetic acid; Bruker Daltonik) (57). MALDI-TOF MS spectra were acquired with a Microflex LT/SH mass spectrometer (Bruker Daltonik) calibrated with the Bruker Bacterial Test Standard in the mass range between 2 and 20 kDa (25). Up to 24 raw spectra were analyzed by the MALDI Biotyper Compass Explorer4.1software (BrukerDaltonik)to generate the reference spectra (MSP—MainSpectraProjection) and a UPGMA dendrogram was created with BioloMICS v12 (BioAware).

IR Biotyper spectrum acquisition and analysis

Overnight cultures of *C. auris* were cultivated on SDA medium. The biomass of two times 10 µL inoculation loops was transferred into 70 µL of 70% (*v*/*v*) ethanol in 1.5 mL tubes. The tubes were equipped with metal rods to allow for better homogenization (Bruker Daltonik). The suspension was homogenized by 5 min vortexing; then, 70 µL of deionized water was added followed by 5 min additional shaking. Fifteen microliters of the suspension was spotted in triplicate on a silicon sample plate (Bruker Daltonik) and dried at 37 °C. The experiment was repeated on three different days to cover cultural variance. Spectra were acquired using the IR Biotyper system v2.1 (Bruker Daltonik) with the following default analysis settings: 32 scans per sample, 10 kHz scan speed, 6 cm^{-1} resolution, Blackman–Harris 3-term apodization, and zero filling 4. Spectra with an absorption < 0.4 or > 2, a signal/noise ratio < 40 in the carbohydrate range, and/or fringes > 10^{-4} were not considered for further analysis as these will not be in agreement to already defined factors to provide the default quality criteria, and may have led to either failed or wrong outcomes (32). The original absorption spectra consisting of

3629 data points over the range from 4000 to 500 cm^{-1} were processed as follows: the second derivative was taken over nine data points, cut to 1300–800 cm^{-1}, and vector-normalized. For each of the isolates, the arithmetic mean of qualified spectra was calculated, so that each mean spectrum was composed of 3–12 single spectra. A UPGMA dendrogram was generated with BioloMICS v12 (BioAware).

Comparison of clustering concordance

Pearson correlation (Mantel test) between the different typing methods was calculated using BioloMICS v12 (BioAware) (58).

Results

Comparison of the five typing methods

Five UPGMA-based dendrograms were obtained from the calculated distance matrices. Cophenetic (Mantel test) clustering (59) allowed for a comparison between the distance matrices underlying the respective clustering analyses. A few isolates used in the current study were previously analyzed by whole genome sequence (WGS) analysis and/or microsatellite analysis (19,39). Comparison of data acquired for those isolates in this study and previous ones showed good agreement between the microsatellite data of the respective analyses (Figure 1, Table 1) (19). Only the isolate from Israel (19,44), that was previously classified by microsatellite typing in clade III, grouped into cluster IV in the current study. For the comparison between the five typing methods addressed here, we considered the microsatellite assay as the reference method for *C. auris* typing. As indicated in the analysis of the single typing approaches (see below), some yielded similar clustering patterns to the microsatellite analysis, whereas others differed widely. In general, cophenetic correlation values were low indicating poor concordance between the methods investigated. Using cophenetic clustering

analysis, microsatellite typing showed the highest agreement with ITS sequencing showing a correlation of 0.45, followed by results from the IR Biotyper with a score of 0.33 (Table 2). The cophenetic correlation between ITS sequence-based typing and results from MALDI-TOF MS was only 0.29. Even lower agreement was observed between microsatellite typing and MALDI-TOF MS with a score of 0.21, and with AFLP with a score of 0.22 (Table 2). The AFLP analysis showed very low scores with typing results obtained from ITS sequence, MALDI-TOF MS, and IR Biotyper analyses, indicating lack of concordance. There was no agreement between MALDI-TOF MS and IR Biotyper as these methods had a similarity score of -0.011 (Table 2).

Table 1. A summary of the clusters and sub-clusters created by the five typing methods used in this study.

	Clusters	Subclusters	NO of isolates (Known cluster names based on microsatellite assay & countries of origin)
Microsatellite assay	Microsatellite I	19 sub-clusters (Ia-n)	61 isolates (Cluster MS I= South Asia/Middle East)
	Microsatellite II	3 sub-clusters (IIa-c)	6 isolates (Cluster MS II= East Asia)
	Microsatellite III	6 sub-clusters (IIIa-f)	19 isolates (Cluster MS III= Israel, South Africa, Spain, Switzerland)
	Microsatellite IV	6 sub-clusters (IVa-b)	10 isolates (Cluster MS IV= Israel, South America)
ITS sequencing	ITS I	NA	80 isolates (Cluster MS I & III= South Africa, South Asia/Middle East, Spain, Switzerland)
	ITS II	NA	6 isolates (Cluster MS II= East Asia)
	ITS III	NA	10 isolates (Cluster MS IV= Israel, South America)
AFLPgenotyping assay	AFLP I	4 sub-clusters (Ia-d)	39 isolates (Cluster MS I, II, III, IV)
	AFLP II	4 sub-clusters (IIa-d)	57 isolates (Cluster MS I, II, III= East Asia, South Asia/Middle East, Spain)
MALDI-TOF MS	MALDI-TOF I	NA	11 isolates (Cluster MS III & Cluster IV= Israel, South America, Spain)
	MALDI-TOF II	NA	77 isolates (Cluster MS I, II, III, Cluster IV)
	MALDI-TOF III	NA	2 isolates (Cluster MS III= South Africa)
	MALDI-TOF IV	NA	2 isolates (Cluster MS II= Korea)
	MALDI-TOF V	NA	2 isolates (Cluster MS I= Oman, Kuwait)
	MALDI-TOF VI	NA	2 isolates (Cluster MS I, II= India, Japan)
IR Biotyper	IR Biotyper I	4 sub-clusters (Ia-d)	78 isolates (Cluster MS I, II, III, IV)
	IR Biotyper II	NA	18 isolates (Cluster MS I, III= India, Kuwait, Malaysia, Spain, Switzerland, Turkey)

Among the 96 tested *C. auris* isolates, isolate number CBS 10913 (Japan) is the type-strain and this strain was included in this study with two other IDs, namely CDC 381 and CWZ 10031064. Additionally, a Korean isolate, namely CBS 12372 (KCTC 17809), was present as duplicate CWZ 10031062. These isolates provided us the capability to test the technical reproducibility of the five typing methods used here. According to the created clusters and sub-clusters, the Japanese triplicate and Korean duplicate isolates correctly grouped into the South East cluster by the microsatellite assay and ITS sequencing. The IR Biotyper typing method classified the triplicated Japanese type-strain into one sub-cluster, whereas the Korean isolates were divided into two sub-clusters. AFLP fingerprinting and MALDI-TOF MS analysis grouped the respective isolates into different clusters and sub-clusters. Furthermore, 10 isolates belonged to the South America cluster and were grouped together with all methods, except MALDI-TOF MS. This method separated one isolate from Venezuela (CDC 386) out of the remaining nine isolates and placed it in cluster MALDI II with isolates from South Asia, East Asia, and South Africa.

Table 2. Correlation between the results obtained with five typing methods used for 96 *Candida auris* isolates. Data were calculated by Pearson correlation between the methods by using BioloMICS v12 software. Green colors show the highest similarities between two methods, followed by orange (middle agreements) and red (lowest agreements). The highest similarity was observed between microsatellite assay and ITS sequencing followed by microsatellite assay and IR Biotyper typing method. The lowest similarity was obtained between microsatellite and MALDI-TOF MS.

Methods	ITS sequencing	MALDI-TOF MS	AFLP	IR Biotyper	Microsatellites
ITS sequencing		0.285	0.065	-0.010	0.446
MALDI-TOF MS	0.285		0.052	-0.011	0.210
AFLP	0.065	0.052		0.061	0.215
IR Biotyper	-0.010	-0.011	0.061		0.330
Microsatellites	0.446	0.210	0.215	0.330	

Microsatellite typing

The microsatellite typing analysis showed four main clusters labeled Microsatellite I-IV (MS I-IV) (Figure 1). Sixty-one isolates (63.5%) clustered in MS I, and included isolates of patients originating from Austria (a Turkish patient), Belgium (Kuwaiti patients), India, Malaysia, Oman, Pakistan, and Saudi Arabia. The East Asia group (cluster II = MS II) included six isolates (6.25%) from Japan and Korea. Cluster MS III had 19 isolates (19.8%), was the second largest group, and contained isolates from South Africa and Spain, but also with two isolates from Israel and Switzerland. Finally, cluster MS IV included 10 isolates (10.4%) from Israel (n = 8) and Venezuela (n = 2). Cluster MS I contained six sub-clusters with strains showing the same microsatellite patterns (Table 1). Sub-cluster MS Ia contained 34 isolates originating from India and Pakistan, but also from the Middle East region. This was also true for sub-cluster MS Ib with six isolates, sub-cluster MS Ie with three isolates, and sub-cluster MS If with five isolates. Remarkably, three isolates from Malaysia were genetically distinct and clustered basally in MS I (MS Il, Im, In). In MS II, the three Japanese isolates were identical (sub-cluster MS IIa), but distinct from the three South Korean isolates (sub-clusters MS IIb and IIc). The duplicated Korean isolates, CBS 12372 = CWZ 10031062, were categorized together in sub-cluster MS IIb. Cluster MS III contained six sub-clusters. Sub-cluster MS IIIb had seven isolates from Spain and South Africa, MS IIIe included four isolates from Spain and Switzerland, MS IIIa had three isolates from Spain, MS IIId had two isolates from Israel and Spain, and MS IIIf contained two isolates from Israel that originated from patients that came from South Africa. Finally, MS IIIc contained only a single Spanish isolate. Cluster MS IV showed six sub-clusters that all had only one or two isolates. Note that the eight Israeli isolates belonging to five sub-clusters of MS IV were genetically distinct from those belonging to clusters MS IIId and MS IIIf. The two isolates from Venezuela had the same microsatellite pattern and were distinct from the Israeli isolates in cluster MS IV. To conclude, three of the

four main clusters contained isolates from patients originating from diverse continents (Table 1).

ITS sequence-based typing

A UPGMA analysis using the Pearson correlation algorithm divided the 96 *C. auris* isolates into three clusters, ITS I-III. Eighty isolates (83.3%) including Austria (from a Turkish patient), Belgium (Kuwaiti patients), India, Israel, Malaysia, Oman, Pakistan, Saudi Arabia, South Africa, Spain, and Switzerland had identical ITS sequences. The cluster, ITS I, contained representatives of clusters MS I and MS III as revealed by the microsatellite typing analysis. The second cluster ITS II was fully concordant with cluster MS II, as revealed by microsatellite typing, and had six isolates (6.25%) from Japan and Korea. The remaining group, cluster ITS IV, had the same 10 isolates (10.42%) from Israel and Venezuela as the microsatellite typing identified cluster MS IV (Figure 2, Table 1).

AFLP fingerprinting

AFLP fingerprinting clustered the 96 *C. auris* isolates into two main clusters (Figure 3), and each of these two clusters were divided into four sub-clusters (AFLP Ia-d and AFLP IIa-d). Isolates from Austria (from a Turkish patient), Belgium (Kuwaiti patients), India, Israel, Korea, Malaysia, Oman, Pakistan, and Saudi Arabia belonged to clusters AFLP Ia, Ib, Id, IIa, and IIb. Similarly, isolates from Spain and South Africa belonged to clusters AFLP Ia, Ib, and IIa. Two Israeli isolates with the same microsatellite typing pattern MS IIIf, namely, TAU 171103597 and TAU 171103598 that both came from patients originating from South Africa, clustered in the clusters AFLP Ia and Ib. Cluster MS IV remained intact in the AFLP analysis as cluster AFLP Ic. Indian isolates belonged to cluster MS I and showed quite some genetic divergence by AFLP fingerprinting, indicating that they are not genetically homogeneous. For instance,

isolates from microsatellite cluster MS Ia belonged to clusters AFLP Id, IIa, and IIb (Figures 1 and 3; Table 1). Isolates from MS If clustered into AFLP clusters Ib and Id. The Japanese duplicated type-strains (CBS 10913 and CWZ 10031064) were located in cluster AFLP II (sub-clusters IIc and IId, respectively). In contrast, the third strain from this type strain, namely CDC 381, grouped in cluster AFLP Ia that was distinct from sub-clusters IIc and IId. The duplicated Korean isolates, CBS 12372 = CWZ 10031062, were both in cluster AFLP II, but in different sub-clusters IIc and IId, respectively. Another Korean isolate, CWZ 10031063, was classified into AFLP Id.

MALDI-TOF MS

A dendrogram was generated based on the MALDI-TOF MS MSPs, yielding in two distinct clusters, MALDI I and MALDI II, with eight isolates forming basal lineages without assignment to a cluster. Cluster MALDI I contained isolates from MS III and MS IV, but cluster MALDI II had isolates of all four major microsatellite clusters (MS I-IV) that were previously recognized. The eight basal isolates belonged to clusters MS I, MS II, and MS III. Given the considerable proteomic variation, we refrained from assigning further sub-clusters (Figure 4). Two duplicates of the Japanese type-strain, namely, CBS 10913 and CDC 381, grouped in cluster MALDI II together with a Korean isolate (CBS 12372), while the third type-strain, CWZ 10031064, formed a small group together with the Indian isolate CWZ 10051257. Two other Korean isolates grouped together as sub-cluster MALDI IV.

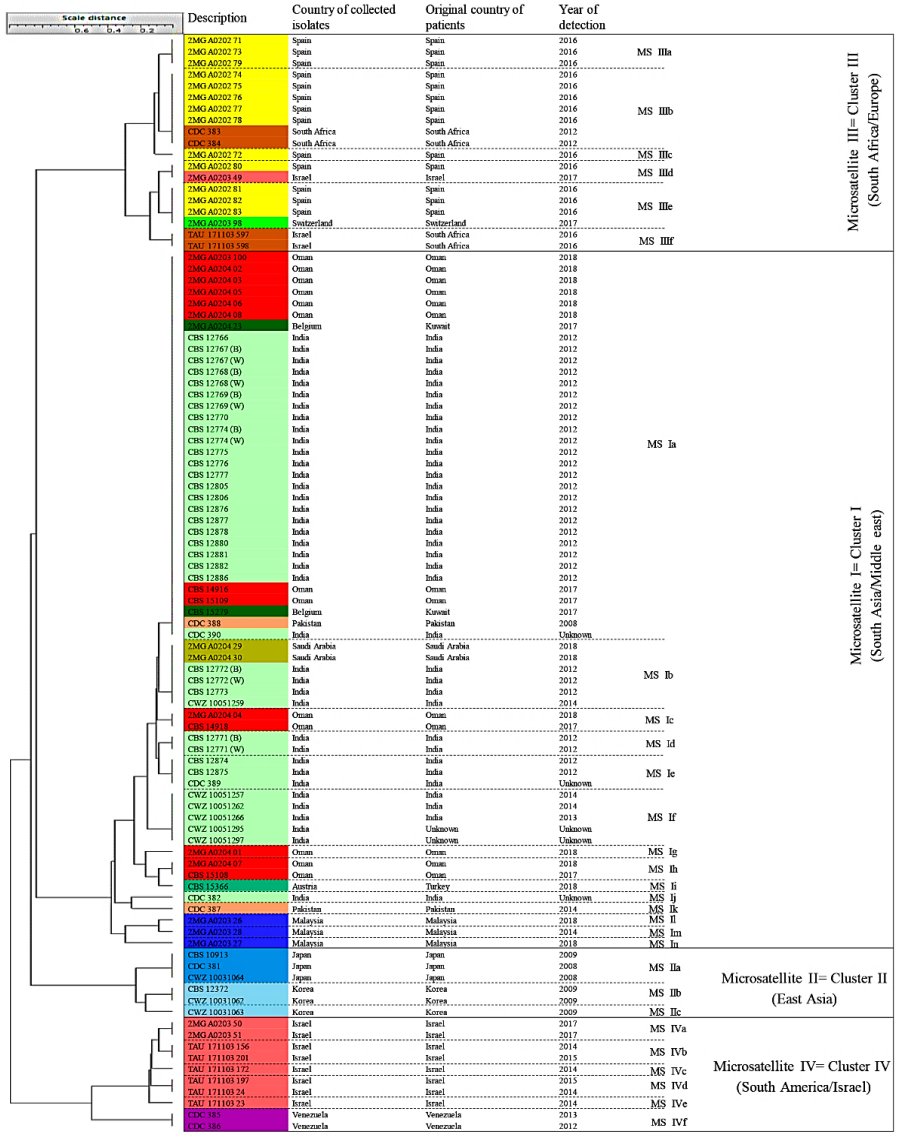

Figure 1. A UPGMA dendrogram was generated with BioloMICS v12 based on microsatellite analysis. Four main geographically linked clusters, namely South Asia/Middle East, East Asia, South Africa/Europe, and South America/Israel were created using 96 isolates. Microsatellite abbreviation (MS) was used for sub-clusters. The color coding used for this dendrogram was the same as follows: Austria (turquoise), Belgium (dark green), Japan (blue), India (light green), Israel (rose), Korea (light blue), Malaysia (dark blue), Oman (red), Pakistan (light pink), Saudi Arabia (chartreuse), Spain (yellow), South Africa (brown), Switzerland (green), and Venezuela (purple).

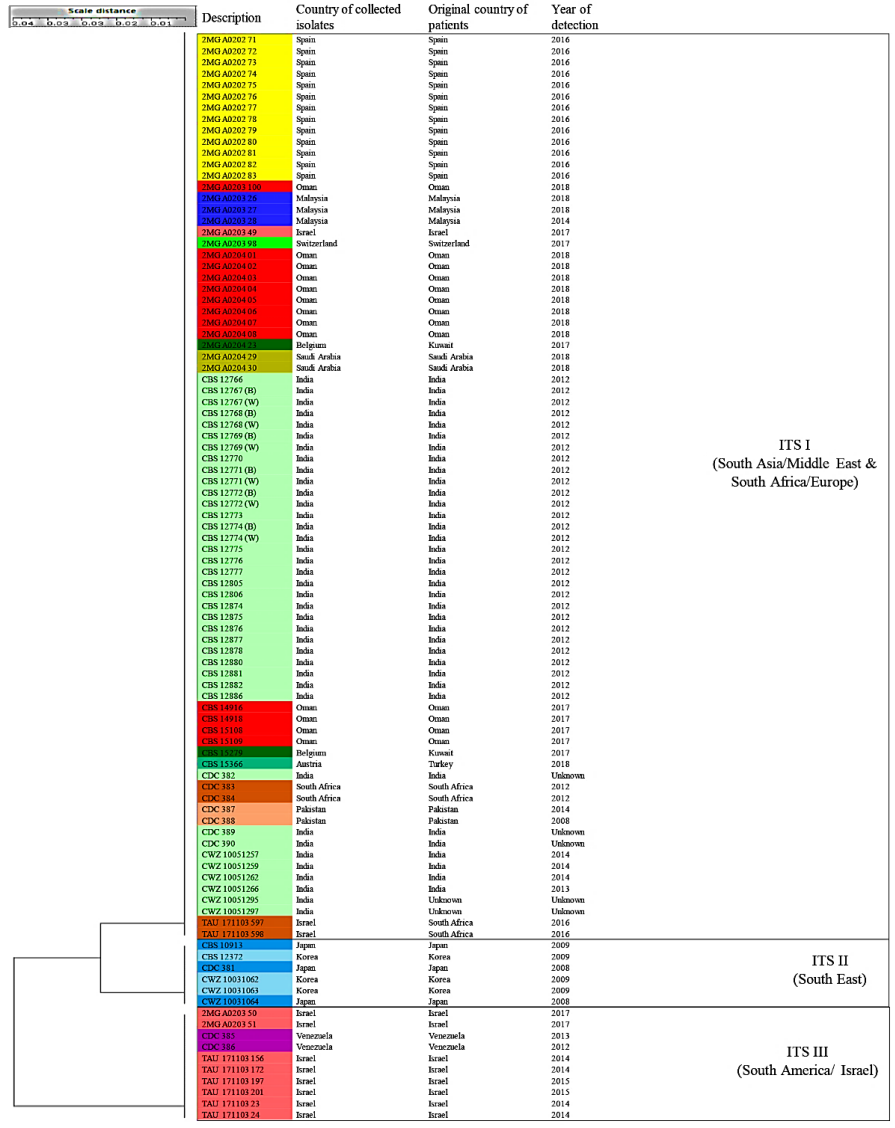

Figure 2. A UPGMA dendrogram generated with BioloMICS v12 made three clusters based on ITS sequences. ITS I included isolates from South Asia/Middle East and South Africa/Europe, ITS II and III included isolates from East Asia and South America/Israel, respectively. The color coding used for this dendrogram was the same as Figure 1.

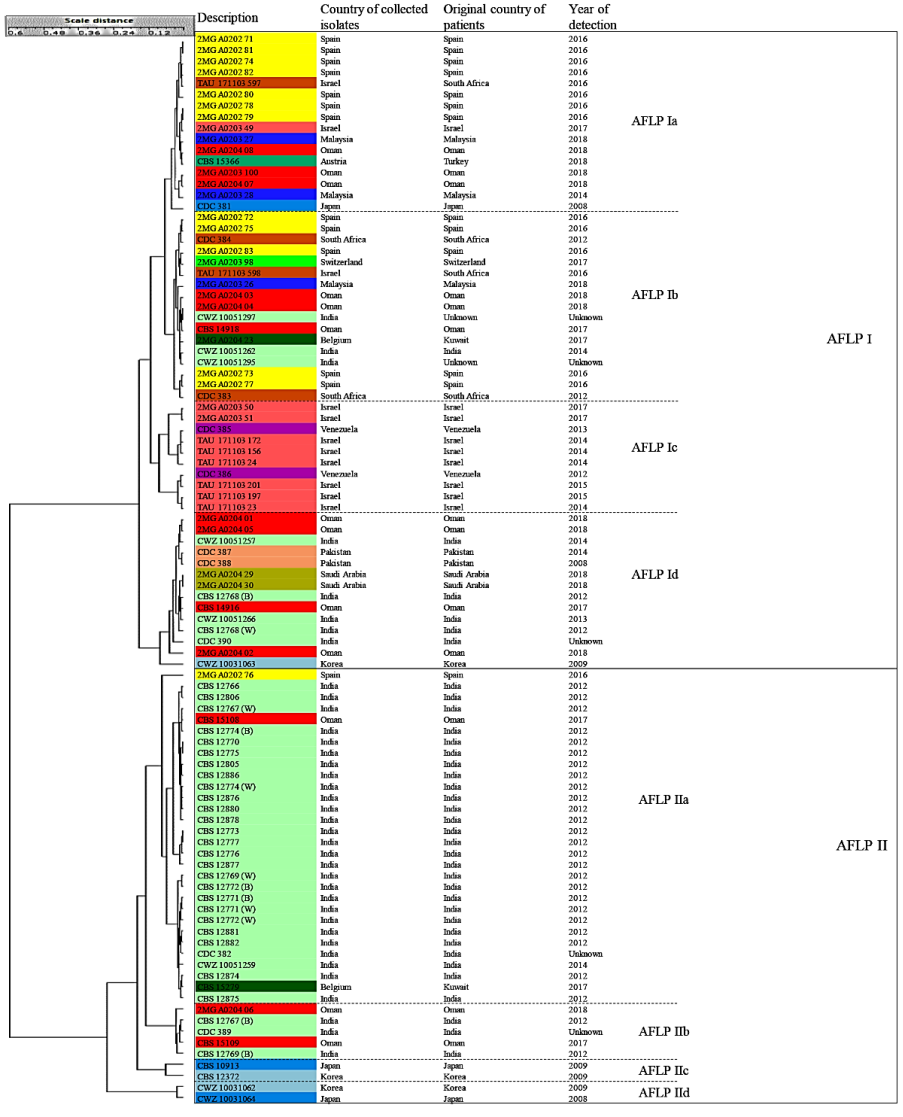

Figure 3. A UPGMA dendrogram was generated with BioloMICS v12 based on AFLP genotyping results. Two main clusters, namely, AFLP I and AFLP II, were created and each was divided into four sub-clusters, AFLP Ia-Id and AFLP IIa-IId. The color coding used for this dendrogram was the same as Figure 1.

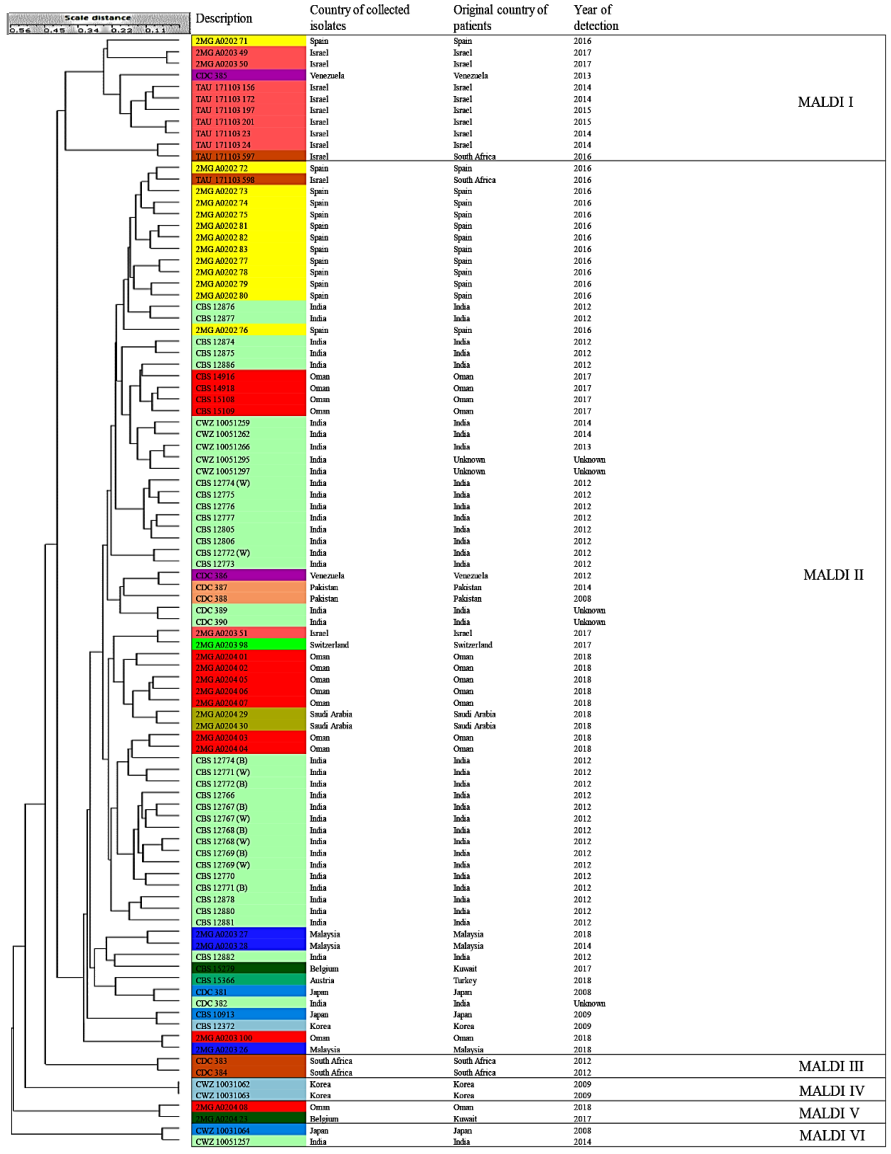

Figure 4. A UPGMA dendrogram was generated with BioloMICS v12 for 96 C. auris isolates by MALDI-TOF MS. Two main clusters were made, namely, MALDI I and MALDI II, with eight isolates forming basal lineages without assignment to a cluster. The color coding used for this dendrogram was the same as Figure 1.

IR Biotyper

In total, 860 IR spectra of 96 *C. auris* isolates were measured. Figure 5 shows that two major clusters can be recognized, namely IR I with 78 isolates (81.25%) and IR II with 18 isolates (18.75%). Similar to the MALDI-TOF MS-based clustering, no concordance was observed between the IR Biotyper clustering and the previously assigned main clusters based on microsatellite typing (Figure 5, Table 1). Isolates of cluster IR I were classified to four sub-clusters (IR Ia-Id) that each agreed with one of four major MS clusters, whereas IR II contained representatives of clusters MS I and MS III. Given the high level of diversity within genotype IR II, we refrained from assigning further subgroups. IR Biotyper clustered all six isolates from East Asia into the IR Ib cluster, excluding the Korean isolate CWZ 10031062 that occurred in the sub-cluster IR Id.

Discussion

This study was undertaken to evaluate the applicability of IR Biotyper as a biochemical typing method to type *C. auris* isolates and compare the outcome with results obtained by MALDI-TOF MS and molecular typing methods. The main goal for the development of the IR Biotyper method was to establish a rapid typing method to investigate the relatedness between isolates involved in nosocomial outbreaks (29,36). In this study, the five typing approaches, IR Biotyper, MALDI-TOF MS, ITS sequencing, AFLP fingerprinting, and microsatellite typing, did not show concordance.

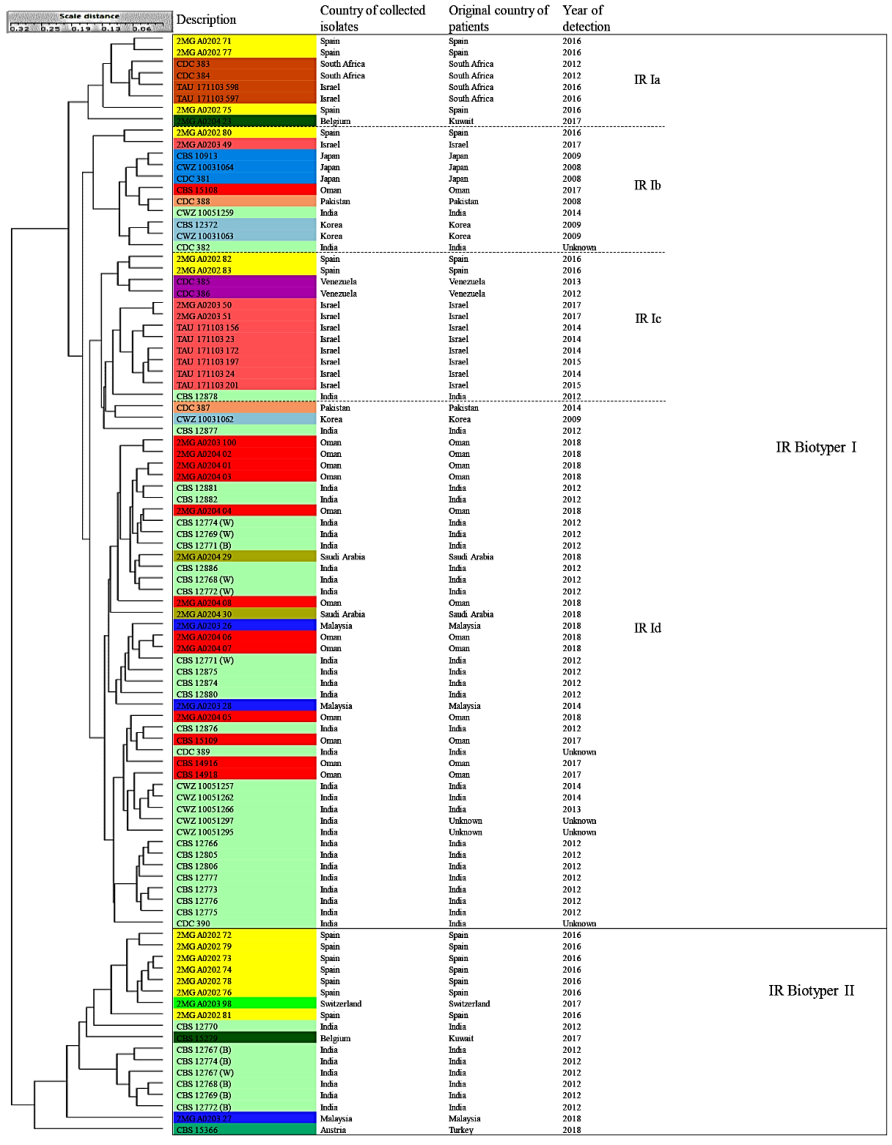

Figure 5. A UPGMA dendrogram generated with BioloMICS v12 of spectra generated by IR Biotyper. Two major clusters were created, namely, IR I and IR II, and among them, cluster IR I was divided into four sub-clusters, IR Ia-Id. IR Biotyper abbreviation (IR) was used for sub-clusters. The colur coding used for this dendrogram was the same as Figure 1.

Microsatellite typing is a rapid molecular typing method used for the analysis of genetic variation in and between fungal populations (21,60) and has been broadly used for typing *Aspergillus flavus*, *Aspergillus fumigatus*, and *Candida* species, such as *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, and *Candida tropicalis*, as well as to investigate three decades of *Cryptococcus neoformans* epidemiology in the Netherlands (20,21,61–64). In this study, the results obtained by microsatellite typing in which four clusters, MS I-IV, were recognized, had the best concordance with typing studies based on WGS analysis (17,39), gene sequencing (18), and a previous microsatellite typing study (19). WGS, as the presumed gold standard, provided background information about the previously geographically defined four clades I-IV. All four WGS CDC reference strains were correctly classified using the microsatellite assay into their respective clusters. The only exception related to cluster MS III that included one Israeli isolate (2MG A0203 49) and one isolate from Switzerland (2MG A0203 98). Since all Israeli isolates were grouped in cluster IV (=South America) with isolates from Venezuela, the history of single Israeli isolate (2MG A0203 49) located in cluster III was investigated. The Israeli patient had a motor vehicle accident in South Africa after which he was repatriated to Israel while infected with *C. auris* (44). The isolate from Switzerland (2MG A0203 98) has not been used in other studies and we could not compare the results; however, the Swiss patient was hospitalized in Geneva after a vacation in northwestern Spain where she was admitted to a local hospital (50). Therefore, the patient's travel history could explain some peculiar results we obtained in our typing studies. Furthermore, the data in this study largely corroborated results from a recent microsatellite typing study that included 444 *C. auris* isolates (Figure 1, Supplementary Table S1) (19). We conclude that microsatellite analysis is a useful and easy-to-use typing method with high reproducibility and portability to understand geographical patterns of *C. auris* populations, and has lower costs compared with WGS (65).

ITS sequencing largely corroborated the clusters found by microsatellite analysis, except for isolates from South Africa and Spain (MS III) that grouped together with isolates from South Asia (MS I) and that clustered differently with microsatellite typing. Thus, ITS sequence-based typing has less resolution than microsatellite analysis. The CDC *C. auris* reference strains, CDC 388 (Pakistan), CDC 381 (Japan), and CDC 385 (Venezuela), were correctly classified into ITS I, ITS II, and ITS IV, respectively, while isolate CDC 383 (South Africa) was grouped incorrectly into ITS I.

AFLP fingerprinting has remained a method with broad applications in outbreak investigations, microbial clustering studies, and typing (18,66–69). This method has high robustness to discriminate between isolates and species; additionally, compared to other typing methods, such as microsatellite typing and WGS analysis, AFLP fingerprinting is cheaper and requires less time. However, its reproducibility remains a problem as data from two experiments (17,19,69–71) cannot be combined. In the AFLP study of Chowdhary et al. (2013), a small sample size (30 isolates) was used to classify *C. auris* isolates according to the respective geographical origin. The isolates from India clustered together, and the isolates from Japan and Korea created another cluster. In their study, AFLP method could successfully classify isolates into two different clusters based on the isolates' geographical origins (72). Next, Prakash et al. (2016) performed a study using a larger sample size of 104 isolates. They focused on isolates from India, Venezuela, and South Africa plus two control isolates from Japan and Korea (18). Their AFLP analysis classified Indian and South American isolates into two major clusters, and the South African isolates were randomly distributed (18). In our study, AFLP categorized isolates into two main clusters (AFLP I and AFLP II) and each one included four sub-clusters. Thus, previously recognized clusters obtained by microsatellite typing and ITS sequencing did not, to a large extent, corroborate with the obtained AFLP clustering. A comparison of AFLP fingerprinting with microsatellite typing indicated that the isolates from South Asia and East

Asia were apparently randomly divided into both the main AFLP clusters, while the isolates from the presumed South African (South Africa and Spain) and South American (Venezuela and Israel) clades were located in cluster AFLP I. The reference isolates CDC 381 (Japan), CDC 383 (South Africa), CDC 385 (Venezuela), and CDC 388 (Pakistan) were distributed into cluster AFLP I (sub-clusters AFLP Ia, AFLP Ib, AFLP Ic, and AFLP Id, respectively).

MALDI-TOF MS is a protein-based method that has been used for *Candida* typing in several studies (31,66,73,74). In the current study, six MALDI-TOF MS clusters were generated and when compared with microsatellite typing, poor agreement was observed between both methods with a correlation of only 0.21. MALDI-TOF MS differentiates between isolates of the same species based on the diversity of the proteome and this may not always provide a similar result of the intrinsic genetic variation of microorganisms due to post-translational modification (PTM) and other experimental variables that influence the protein structure and, consequently, the spectra. Acquired MSPs could be variable due to technical issues, such as accuracy in protein extraction and pipetting samples onto a polished steel target plate. Alternatively, MSP differences could be problematic because they were calculated based on standard MSP settings, which are optimized for species differentiation, not for cluster analysis. Thus, MALDI-TOF MS may not be useful to differentiate between isolates belonging to the same species, as relatively few peaks with differences are generated. Indeed, the gross similarity between the spectra may obviate its utility for intra-species typing (18,73,75,76). The MSP-based typing method generates a dendrogram based on log(scores) obtained for each isolate individually and compares them with scores obtained for the other isolates. If there is no significant variation between the log(scores), which might be the case for clonally related isolates, the MSP-based dendrogram analysis may not produce an accurate result (18,75). Moreover, the MSP-based dendrogram should always contain an outlier isolate, because without such an outlier the dendrogram may result in over-interpretation. For instance, using

some *Candida albicans* and *Candida glabrata* isolates as outliers might affect the acquired results differently. The lack of an outlier species in this study may explain why the MALDI-TOF MS-based dendrogram differed widely from the clustering obtained by microsatellite analysis. The use of BioloMICS v12 (BioAware) to generate a UPGMA dendrogram might not have been the optimal analysis method for the acquired MSPs generated by MALDI-TOF MS. Other available analytical methods, such as BioNumerics, and a comparison between dendrograms created by these different approaches may improve the analytical outcomes generated as MSP-based dendrograms. Four MALDI-TOF MS clusters, MALDI III-VI, each containing only two isolates, were generated that occurred distantly from the main MALDI-TOF MS clusters (MALDI I and MALDI II). The reference strain CDC 385 (Venezuela) clustered in MALDI I that included isolates from Israel and Venezuela (=cluster MS IV = South American clade); isolate CDC 383 (South Africa) created a small group with another isolate from the same region (CDC 384) that belonged to the South Africa cluster; isolates CDC 381 (Japan) and CDC 388 (Pakistan) were located in the MALDI II cluster that included isolates from MS I, MS II, MS III, and MS IV. Overall, the MALDI Biotyper provides excellent performance for species identification; however, it is not useful for typing different *C. auris* strains when compared to the microsatellite typing assay.

The acquisition of IR Biotyper spectra depends highly on the conditions used during culturing of the yeasts (32,77). The method is rapid, providing final results in 1–2 h per sample and is easy to use without any prior knowledge of FTIR technology. This method has been developed with a possibility to change different parameters during the analysis to obtain different clustering patterns. Using the IR Biotyper method, each isolate was measured nine times and the final result was normalized by merging the nine acquired spectra. Consequently, a single misclassification due to the impact of one of the variable factors should not affect the clustering. Moreover, by measuring every isolate nine times in this manner, the reproducibility of the IR

Biotyper method increases in microbial typing according to the manufacturer's recommendations. In this study, IR Biotyper analysis created two main clusters, namely IR I and IR II. Comparing IR Biotyper and microsatellite typing results revealed an agreement with a correlation score of 0.33, which ranked IR Biotyper as showing the second-highest agreement with microsatellite typing after ITS sequencing (Table 2). Two Israeli isolates that originated from South Africa (TAU 171103 597 and TAU 171103 598) were placed in sub-cluster IR Ia (cluster III = South Africa), which was in agreement with the microsatellite typing analysis, and supported the findings by Belkin et al. (2017) (44) who reported that the Israeli *C. auris* isolates originally came from South Africa. Isolate numbers CDC 381 (Japan) and CDC 388 (Pakistan) grouped in the major cluster IR I (sub-cluster IR Ib) with isolates from India, Israel, Oman, and Spain. CDC 383 (South Africa) and CDC 385 (Venezuela) grouped in sub-clusters IR Ia and IR Ic and belonged to cluster MS III (South Africa) and MS IV (South America), respectively. Thus, more effort is required to evaluate and optimize several parameters in measuring and analyzing data obtained by FTIR spectroscopy before this method can be used to investigate intra-species types in hospital outbreaks caused by *C. auris* and, likely, , isolates of other yeast species. A further analysis was performed to evaluate the reproducibility of the five typing methods investigated in this study using triplicated *C. auris* Japanese type-strain, and duplicate Korean isolates. The observed result after microsatellite and ITS typing showed the high technical reproducibility of both methods. The IR Biotyper method indicated reliable reproducibility by clustering all Japanese type-strains into one sub-cluster. In contrast, AFLP fingerprinting and MALDI-TOF MS analysis placed the triplicated Japanese and duplicate Korean isolates in non-concordant subgroups that referred to their low power of technical reproducibility.

In summary, none of the typing methods explored in this study showed a concordance above 50%. Comparing the results obtained by the microsatellite typing assay, the other methods

created different clusters or sub-clusters, including separating or combining isolates from different countries and continents. This observation is difficult to explain, but lack of information about the patient's background may impact our understanding of the epidemiology of *C. auris* infections. In addition, this result may also be due to intrinsic genetic, biochemical, or phenotypic variations among *C. auris* isolates. Genomic variation can be generated via small local alteration of the nucleotide sequence of the genome, intragenomic restructuring, and the acquisition of genetic material from other *Candida* species or even other microbes to adapt to different environmental conditions as well as the human body (78–80). A recent study based on a WGS analysis showed limited genetic diversity between isolates within each of the four *C. auris* clades, whereas genetic diversity was obvious between the four clades (17). All analyses in our study, except the results from the microsatellite typing method, showed extensive variation, but no clear clustering according to the previously defined four main clades. As an exception, eight out of ten South American (=clade IV) isolates showed an identical clustering in all five typing methods used in this study that were largely in agreement with results obtained from other typing studies (Supplementary Table S1). Taken together, to obtain a correct insight into the epidemiological distribution of *C. auris*, further analyses based on a larger and blinded sample size and inclusion of various independently working laboratories, using WGS as a reference, are needed to evaluate other less costly methods as explored here.

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Conflict of interest: M.K., N.M., T.M., and M.V. are employees of the mass spectrometry company Bruker Daltonik GmbH. All authors declare that they have no competing interests. V.R. is an employee of the WI and CEO of BioAware and has no competing interest.

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Supplementary Material Table 1. Overview of 96 *Candida auris* strains used in this study is shown in this table. The only discrepancy between the clusters found by other studies and the microsatellite in this study is highlighted by red color. Isolates marked with purple color belonged to cluster IV (South America clade) have shown identical clustering in all five typing methods used in this study compared to the clusters (=clades) obtained from other studies based-molecular techniques. This shows that these isolates are highly genetically identical, but contrary, there is an obvious genetic diversity between these isolates compared to those from the other three clades.

Other names	ID (Used in our study)	Country of collected isolates	Original country of patients	Clades (Clusters)-According other studies (WGS, ITS, Microsatellite)[1,2,3]	Clusters-According to Microsatellite results in this study	Year of detection (Identification)
B11220	CDC 381 [b]	Japan	Japan	Cluster II (East Asia clade)	Cluster MS II (East Asia)	2008
	CDC 382	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	Unknown
B11221	CDC 383	South Africa	South Africa	Cluster III (South Africa clade)	Cluster MS III (South Africa/ Europe)	2012
B11222	CDC 384	South Africa	South Africa	Cluster III (South Africa clade)	Cluster MS III (South Africa/ Europe)	2012
B11243	CDC 385	Venezuela	Venezuela	Cluster IV (South America clade)	Cluster MS IV (South America/ Israel)	2013
B11244	CDC 386	Venezuela	Venezuela	Cluster IV (South America clade)	Cluster MS IV (South America/ Israel)	2012
B11098	CDC 387	Pakistan	Pakistan	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2014
B8441	CDC 388	Pakistan	Pakistan	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2008
	CDC 389	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	Unknown
	CDC 390	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	Unknown
B11894	TAU-171103-23	Israel	Israel	Cluster IV (South America clade)	Cluster MS IV (South America/ Israel)	2014
B11896	TAU-171103-24	Israel	Israel	Cluster IV (South America clade)	Cluster MS IV (South America/ Israel)	2014
B11892	TAU-171103-156	Israel	Israel	Cluster IV (South America clade)	Cluster MS IV (South America/ Israel)	2014
B11893	TAU-171103-172	Israel	Israel	Cluster IV (South America clade)	Cluster MS IV (South America/ Israel)	2014
B11895	TAU-171103-197	Israel	Israel	Cluster IV (South America clade)	Cluster MS IV (South America/ Israel)	2015
B11897	TAU-171103-201	Israel	Israel	Cluster IV (South America clade)	Cluster MS IV (South America/ Israel)	2015
B11223	TAU-171103-597	Israel	South Africa	Cluster III (South Africa clade)	Cluster MS III (South Africa/ Europe)	2016
B11224	TAU-171103-598	Israel	South Africa	Cluster III (South Africa clade)	Cluster MS III (South Africa/ Europe)	2016
B11808 (KCTC 17809)	CWZ-10031062	Korea	Korea	Cluster II (East Asia clade)	Cluster MS II (East Asia)	2009
B11809 (KCTC 17810)	CWZ-10031063	Korea	Korea	Cluster II (East Asia clade)	Cluster MS II (East Asia)	2009
B11220 (CDC 381)	CWZ- 10031064 [b]	Japan	Japan	Cluster II (East Asia clade)	Cluster MS II (East Asia)	2008
VPCI 467/P/14	CWZ-10051257	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2014
VPCI 471A/P/14	CWZ-10051259	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2014
VPCI 478/P/14	CWZ-10051262	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2014
VPCI 1133/P/13	CWZ-10051266	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2013
	CWZ-10051295	India	Unknown	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	Unknown
	CWZ-10051297	India	Unknown	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	Unknown
05-299	2MG-A0204-23	Belgium	Kuwait	Cluster I (South Asia/Middle East clade)	Cluster MS I (South Asia/Middle East)	2017
Repeat of 05-299	CBS 15279	Belgium	Kuwait	Cluster I (South Asia/Middle East clade)	Cluster MS I (South Asia/Middle East)	2017
JCM 15448	CBS 10913 [b]	Japan	Japan	Cluster II (East Asia clade)	Cluster MS II (East Asia)	2009
KCTC 17809	CBS 12372	Korea	Korea	Cluster II (East Asia clade)	Cluster MS II (East Asia)	2009
VPCI669/P/12	CBS 12766	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI673/P/12	CBS 12770	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI 677/P/12	CBS 12773	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI 709/P/12	CBS 12775	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI711/P/12	CBS 12776	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI 712/P/12	CBS 12777	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI 683/p/12	CBS 12805	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI 692/p/12	CBS 12806	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI473	CBS 12876	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI474	CBS 12877	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
	CBS 14916	Oman	Oman	Cluster I (South Asia/Middle East clade)	Cluster MS I (South Asia/Middle East)	2017
	CBS 14918	Oman	Oman	Cluster I (South Asia/ Middle East clade)	Cluster MS I (South Asia/Middle East)	2017
317062126	CBS 15108	Oman	Oman	Cluster I (South Asia/ Middle East clade)	Cluster MS I (South Asia/Middle East)	2017
317052804	CBS 15109	Oman	Oman	Cluster I (South Asia/ Middle East clade)	Cluster MS I (South Asia/Middle East)	2017
VPCI670/P/12	CBS 12767 (B)	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI670/P/12	CBS 12767 (W)	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI671/P/12	CBS 12768 (B)	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI671/P/12	CBS 12768 (W)	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI672/P/12	CBS 12769 (B)	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI672/P/12	CBS 12769 (W)	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI674/P/12	CBS 12771 (B)	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI674/P/12	CBS 12771 (W)	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI676/P/12	CBS 12772 (B)	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI676/P/12	CBS 12772 (W)	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI708/P/12	CBS 12774 (B)	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI708/P/12	CBS 12774 (W)	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
	CBS 15366	Austria	Turkey	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2018
VPCI471	CBS 12874	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI472	CBS 12875	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI475	CBS 12878	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI477	CBS 12880	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI478	CBS 12881	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI479	CBS 12882	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
VPCI483	CBS 12886	India	India	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2012
	2MG-A0202-071	Spain	Spain	Cluster III (South Africa clade)	Cluster MS III (South Africa/ Europe)	2016
	2MG-A0202-072	Spain	Spain	Cluster III (South Africa clade)	Cluster MS III (South Africa/ Europe)	2016
	2MG-A0202-073	Spain	Spain	Cluster III (South Africa clade)	Cluster MS III (South Africa/ Europe)	2016
	2MG-A0202-074	Spain	Spain	Cluster III (South Africa clade)	Cluster MS III (South Africa/ Europe)	2016
	2MG-A0202-075	Spain	Spain	Cluster III (South Africa clade)	Cluster MS III (South Africa/ Europe)	2016
	2MG-A0202-076	Spain	Spain	Cluster III (South Africa clade)	Cluster MS III (South Africa/ Europe)	2016
	2MG-A0202-077	Spain	Spain	Cluster III (South Africa clade)	Cluster MS III (South Africa/ Europe)	2016
	2MG-A0202-078	Spain	Spain	Cluster III (South Africa clade)	Cluster MS III (South Africa/ Europe)	2016
	2MG-A0202-079	Spain	Spain	Cluster III (South Africa clade)	Cluster MS III (South Africa/ Europe)	2016
	2MG-A0202-080	Spain	Spain	Cluster III (South Africa clade)	Cluster MS III (South Africa/ Europe)	2016
	2MG-A0202-081	Spain	Spain	Cluster III (South Africa clade)	Cluster MS III (South Africa/ Europe)	2016
	2MG-A0202-082	Spain	Spain	Cluster III (South Africa clade)	Cluster MS III (South Africa/ Europe)	2016
	2MG-A0202-083	Spain	Spain	Cluster III (South Africa clade)	Cluster MS III (South Africa/ Europe)	2016
UZ495-18	2MG-A0203-026	Malaysia	Malaysia	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2018
UZ681-18	2MG-A0203-027	Malaysia	Malaysia	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2018
UZ1447-14	2MG-A0203-028	Malaysia	Malaysia	Cluster I (South Asia clade)	Cluster MS I (South Asia/Middle East)	2014
	2MG-A0203-049	Israel	Israel	Cluster IV (South America clade)	Cluster MS III (South Africa/ Europe)	2017
	2MG-A0203-050	Israel	Israel	Cluster IV (South America clade)	Cluster MS IV (South America/ Israel)	2017
	2MG-A0203-051	Israel	Israel	Cluster IV (South America clade)	Cluster MS IV (South America/ Israel)	2017
SMW.2018.14622	2MG-A0203-098	Switzerland	Switzerland	NA	Cluster MS III (South America/ Israel)	2017
	2MG-A0203-100	Oman	Oman	Cluster I (South Asia/ Middle East clade)	Cluster I (South Asia/ Middle East)	2018
	2MG-A0204-001	Oman	Oman	Cluster I (South Asia/ Middle East clade)	Cluster I (South Asia/ Middle East)	2018
	2MG-A0204-002	Oman	Oman	Cluster I (South Asia/ Middle East clade)	Cluster I (South Asia/ Middle East)	2018
	2MG-A0204-003	Oman	Oman	Cluster I (South Asia/ Middle East clade)	Cluster I (South Asia/ Middle East)	2018
	2MG-A0204-004	Oman	Oman	Cluster I (South Asia/ Middle East clade)	Cluster I (South Asia/ Middle East)	2018
	2MG-A0204-005	Oman	Oman	Cluster I (South Asia/ Middle East clade)	Cluster I (South Asia/ Middle East)	2018
	2MG-A0204-006	Oman	Oman	Cluster I (South Asia/ Middle East clade)	Cluster I (South Asia/ Middle East)	2018
	2MG-A0204-007	Oman	Oman	Cluster I (South Asia/ Middle East clade)	Cluster I (South Asia/ Middle East)	2018
	2MG-A0204-008	Oman	Oman	Cluster I (South Asia/ Middle East clade)	Cluster I (South Asia/ Middle East)	2018
	2MG-A0204-029	Saudi Arabia	Saudi Arabia	Cluster I (South Asia/ Middle East clade)	Cluster I (South Asia/ Middle East)	2018
	2MG-A0204-030	Saudi Arabia	Saudi Arabia	Cluster I (South Asia/ Middle East clade)	Cluster I (South Asia/ Middle East)	2018

a. All tested isolates were identified by MALDI-TOF MS.

b. Type-strain.

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Chapter 7

Detection of *Candida* species in blood with the antimicrobial peptide hLF(1-11) using MALDI-TOF MS and fluorescent microscopy

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Abstract

Candida species are responsible for about 40% of all cases of invasive fungal infections worldwide. Sepsis due to invasive candidiasis has a high morbidity and mortality, and therefore the pathogens need to be rapidly identified. In this study, we explored novel methods to detect *Candida* species using a synthetic antimicrobial hLF(1-11) peptide. The hLF(1-11) peptide was labeled either with ^{99m}Tc or fluorescein isothiocyanate (FITC) to determine the binding to yeast cells in whole blood. For this purpose, we employed fluorescence microscopy, radioactive counting, and MALDI-TOF MS for the detection of *Candida albicans* in whole blood. Binding to *C. albicans* cells was demonstrated with ^{99m}Tc-labeled hLF(1-11) showing high sensitivity to *Candida* cells with less affinity to human blood cells. In addition, the results with fluorescence microscopy showed high sensitivity and specificity of the FITC-hLF(1-11) peptide binding to various *Candida* species within 15 min incubation time. In whole blood, *C. albicans*, *C. glabrata*, *C. auris* and various bacteria bound to hLF(1-11) and FITC-hLF(1-11) were identified at low cell concentrations by MALDI-TOF MS. This study support the use of hLF(1-11) as a new tool for the detection of *Candida* and bacterial cells in human blood samples.

Key words: *Candida* identification, hLF(1-11) peptide, MALDI-TOF MS, Immunofluorescent microscopy

Introduction

Bloodstream infections (BSI) due to bacteria and yeasts, such as *Candida* species, are a serious threat to human health (1, 2). Delay in treatment leads to spread of the infection through the body and may cause tissue damage, organ failure, and eventually death (1, 3). BSI occurs in all types of patients, but immunocompromised individuals and patients in intensive care units (ICU) pose a high risk with mortality values ranging between 23 to 50% (4–6). Sepsis and septic shock due to *Candida* species are mostly reported from ICU patients and different factors play a role in the transition of commensal yeasts into an invasive state. *Candida albicans* is known as the clinically most important *Candida* species with a high mortality rate of 40% in BSI (7–10). The increasing incidence of non-*albicans Candida* (NAC) species has changed the epidemiology and outcomes of invasive candidiasis (11). Accordingly, early and rapid identification is important to decide on proper treatment and to reduce morbidity and mortality. The diagnosis of fungal BSI is not easy. First, obtaining a positive blood culture is important and this may take days. Additionally, a number of ICU patients with BSI may have negative culturing results due to use of antifungal therapy (12–16). Furthermore, it is of utmost importance to shorten the time to diagnosis to decide for the most effective treatment options. Secondly, fungi involved in BSI are difficult to detect because of their low cell numbers present in the blood at the onset of infections and there might be overgrowth by co-infecting bacterial species (17, 18). Management of patients hitherto depends on getting a positive blood culture followed by identification using a fast and reliable detection method to identify the microorganisms at the species level (17, 19–21). Presently, there is no generally accepted method to identify bacteria and fungi directly in patients' blood samples. Several efforts have focused on developing a method to enrich, detect and identify microorganisms directly from blood samples, such as use of an immuno-detection platform with intrinsic signal current amplification, a combination of Matrix-Assisted Laser Desorption Ionization–Time of Flight

Mass Spectrometry (MALDI-TOF MS) and/or molecular techniques. MALDI-TOF MS, immuno-affinity enrichment/separation and PCR followed by electrospray ionization mass spectrometry (PCR/ESI-MS) may contribute considerably to reduce the time to effective treatment (19, 22–24). A rapid method that increases the sensitivity and specificity to detect blood borne pathogens, like *Candida* yeasts, is of great importance. For this purpose, an 11 amino acid peptide derived from the N-terminus of the human lactoferrin protein, hLF(1-11), is of potential interest (25). This peptide has been used in pre-clinical settings as a radiotracer to detect infections, but it also acts as an antimicrobial agent (25–28). Due to the rising incidence of *Candida* sepsis (7), the applicability of hLF(1-11) to bind to pathogenic yeast cells is explored in order to see if it can be applied to detect and identify sepsis caused by *Candida* species. In the current study, we evaluated the suitability for using hLF(1-11) as a marker for blood borne pathogens. The binding of hLF(1-11) to *Candida* and bacterial cells was evaluated by using labeled hLF(1-11) either by ^{99m}Tc or fluorescein isothiocyanate (FITC) using radioactivity counting's and fluorescence microscopy, respectively. The identification of *Candida* and bacterial species bound to hLF(1-11) was performed by MALDI-TOF MS from plate agar and in whole blood. For this purpose, hLF(1-11) alone and labeled with the fluorescent marker FITC-hLF(1-11) were used. The results showed high sensitivity binding of labeled hLF(1-11) either by ^{99m}Tc or fluorescein isothiocyanate (FITC) to *Candida* cells with less affinity to human blood cells. Additionally, identification of *C. albicans*, *C. glabrata*, *C. auris*, and various bacteria bound to hLF(1-11) and FITC-hLF(1-11) was successfully performed by MALDI-TOF MS in whole blood samples.

Materials and methods

Isolates and media

Twelve *Candida* isolates belonging to the five clinically most relevant species were used and includes: *C. albicans* CBS 562 (ATCC 18804), CBS 2312 (ATCC 28776), CBS 8758; *C. glabrata* CBS 138 (ATCC 2001), CBS 6144 (ATCC 28482); *C. parapsilosis* CBS 604 (ATCC 22019), CBS 11301; *C. tropicalis* CBS 94 (ATCC 750), CBS 6320 (ATCC 28142); and *C. auris* CBS 10913, CBS 15366, and CBS 15603, obtained from the CBS culture collection hosted at the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands. A selected group of clinically obtained *Candida* isolates from the University Medical Center Utrecht (UMCU), namely six *C. albicans*, three *C. glabrata*, three *C. tropicalis*, and two *C. parapsilosis,* was tested in this study. The bacterial reference species *Escherichia coli* NCCB 100297 (ATCC 25922), *Pseudomonas aeruginosa* NCCB 89161 (ATCC 27853) and *Staphylococcus aureus* NCCB 100294 (ATCC 25923) were used as a control to evaluate the specificity of hLF(1-11) for *Candida* isolates. All isolates were identified by MALDI Biotyper (Bruker Daltonik GmbH, Bremen, Germany) before the start of the experiments. The isolates were stored at 4°C, and fresh overnight cultures were used for the experiments that were grown at 37 °C on Glucose Yeast Peptone Agar (GYPA) and tryptic soy agar (TSA) for *Candida* and bacteria, respectively.

Synthetic peptide structure

Two different peptides were tested to evaluate their use in the detection of microbial pathogens. The hLF(1-11) [GRRRRSVQWCA-NH_2; Mw. 1,415 Da; purity of 98.54%] was purchased from ProteoGenix (Schiltigheim, France). A modified hLF(1-11) labeled with FITC [FITC-Ahx-RRRRSVQWCK-NH_2; Mw. 1,876 Da, purity of >95%], FITC-hLF(1-11), was prepared

by the Laboratory of Organic Chemistry Wageningen University & Research (Wageningen, The Netherlands).

Mass spectrometry for peptide characterization

A Bruker Microflex MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) was used for the analysis of the mass and the presence of peptides (in 49.9% v/v acetonitrile, 49.9% v/v water and 0.2% v/v trifluoracetic acid) in samples. A hLF(1-11) peptide (Mw 1423.7 or dimer version with Mw. 1897.1 Da) purchased from Pepscan (Lelystad, The Netherlands) was run simultaneously as reference peptide. Sample size was 1 µL of an hLF(1-11) peptide solution. Stocks of the peptides were dried in a Speed-Vac (Savant Instruments Inc., Farmingdale, NY, USA) and stored at -70°C prior to use. For the assays, peptides were dissolved in 10 mM sodium phosphate buffer (NaPB) with 0.01% acetic acid (HAc; pH 3.7) to a concentration of 1 mgmL^{-1}.

***In vitro* assay for the binding of ^{99m}Tc-labeled cationic peptides**

In vitro cell binding assays were performed to assess possible relationships between binding capacity and various cell types, including blood cells and pathogens. To avoid any effects of direct metabolism including the interaction with the binding of peptides to *Candida* and/or human blood cells, these experiments were performed at 4°C. The peptide was radioactively labeled with ^{99m}Tc and used in an *in vitro* binding assay as described elsewhere (29, 30). Briefly, 10 µL of a peptide stock solution (1 mg mL^{-1} into 0.01% acetic acid) was added to 4 µL of an aseptic mixture of 950 mg/L $Sn(Cl)_2 \cdot 2H_2O$ and 2 g/L sodium pyrophosphate $10H_2O$ in saline (Dept. Clinical Pharmacy and Toxicology, Leiden University Medical Center, Leiden, The Netherlands). Immediately, thereafter, 2 µL of 10 mg mL^{-1} of KBH_4 (crystalline, Sigma

Chemical, St. Louis, MO, USA) in 0.1 M NaOH were added to this mixture. After the addition of 0.1 mL of ^{99m}Tc-sodium pertechnetate (200 ± 5 MBq/mL MBq/mL, Technekow, Mallinckrodt Medical BV, Petten, The Netherlands) the mixture was gently stirred at room temperature for 1h prior further use. Those preparations will be further referred to as ^{99m}Tc-hLF(1-11).

Binding of blood cells with ^{99m}Tc-hLF(1-11)

Three concentrations of ^{99m}Tc-hLF(1-11) were used, namely 2.5, 5 and 10 µg mL^{-1}, to show the affinity of this peptide to various blood cells. Whole blood was obtained from healthy volunteers. Informed consent was obtained from all subjects in accordance with the Declaration of Helsinki. Approval was obtained from the medical ethics committee of the University Medical Centre Utrecht (METC-protocol 07-125/C approved March 01, 2010; Utrecht, The Netherlands). Briefly, 0.8 mL of the incubation buffer (0.05 M PBS containing 0.1% v/v Tween 80 and 0.05% v/v acetic acid, pH 5), was added to 0.1 mL of the preparation containing the labeled cationic peptide and 0.1 mL of whole blood. A Ficoll gradient of 90% was used for separation of the blood components, such as plasma and various blood cells, after centrifugation for 5 min at 10,000×*g* at 4ºC. The mixtures containing all compounds and cell fractions were gently mixed by vortex and incubated for 1 h at 4°C in sterile low-binding Eppendorf tubes. The radioactivity of the fractions was determined using a dose calibrator (VDC 101, Veenstra Instruments, Joure, The Netherlands). The radioactivity related with *Candida* was expressed as percentage of added ^{99m}Tc activity bound to the various cells and fractions. Tests were independently performed in triplicate in three sets of experiments.

Binding of ^{99m}Tc-hLF(1-11) with *Candida* cells

Briefly, 0.1 ml of PBS containing 1/10 (10 µM) of the mixture of ^{99m}Tc-hLF(1-11) was transferred into an low-binding Eppendorf vial with 0.9 mL of 50/50% (v/v) of HAc/PBS and 0.01% (v/v) Tween 80 plus approximately 2×10^7 viable *Candida* cells, the concentration of which was determined by a Bürker count chamber. The mixture, with a final pH of 5 was incubated for 1h at 4°C and, thereafter, the vials were centrifuged at 2,000×*g* for 5 min at 4°C. The supernatant was removed and the pelleted micro-organisms were gently resuspended in 1 mL of PBS and re-centrifuged as above. The supernatant was removed and the radioactivity in the pellet was determined in a VDC 101 dose calibrator (Veenstra Instruments). The radioactivity related to the binding of the peptide to *Candida* cells was expressed as percentage of added ^{99m}Tc activity bound to 2×10^7 of viable *Candida* cells. Calculated values were corrected for non-specific binding by subtracting with values obtained from incubations without *Candida* cells. Tests were independently performed in three sets of three experiments.

Blood lysis and fluorescence microscopy

In order to improve yeast cell detection using hLF(1-11) directly in blood samples, we investigated possibilities to remove the blood cells using a method without impacting on the cell wall structure of the *Candida* cells. We aimed to develop an in-house blood lysis method to be applicable in laboratories without requiring expensive compounds. The method must have less hands-on-work, to be cost-effective, and able to remove entire blood cells rapidly. Therefore, several trials and errors were performed to find out the best lysis method to fulfill all the above-mentioned factors. Accordingly, the best results were obtained using the developed protocol that will be described as follows. A concentration of 10^5 *Candida* cells in 1 mL of 1× PBS pH 7.4 (Westburg, Leusden, The Netherlands) was prepared using a microplate reader (SPECTROstarNano, BMG LABTECH, Offenburg, Germany). Blood samples were

acquired from healthy donors and spiked with the respective *Candida* species prepared in PBS to a final concentration of 10^4 yeast cells per mL of a blood sample. Two ml of the spiked blood sample was centrifuged at 14,000×g for 10 min (Eppendorf, Centrifuge 5430 R, Hamburg, Germany), and the pellet was rinsed in 1 mL H_2O and centrifuged at 14,000×g for 5 min. The supernatant was removed, and the pellet rinsed in 1 mL 10% NACL (vol/vol) and centrifuged at 14,000×*g* for 5 min. Next, the pellet was rinsed 3 times in 1 mL H_2O and 1× PBS pH 7.4 in tandem to remove blood cells and remain intact yeast cells. The pellet including intact yeast cell was used for evaluation by fluorescence microscopy.

After blood lysis, the acquired pellets containing *Candida* cells were used to make a final concentration of 10^3 *Candida* cells in 300 µL of RPMI 1640 with L-glutamine and sodium bicarbonate (R8757, Sigma-Aldrich). Then, cells were mixed with 10 µL of FITC-hLF(1-11) (0.03 µg mL^{-1}) and incubated for 15 min at room temperature in a ThermoMixer (HLC by DITABIS, Pforzheim, Germany) under agitation at 300 rpm. To remove the excess of labeled peptides, the cells were 10 min centrifuged at 14,000×*g* followed by three washing steps with 1× PBS pH 7.4 with 5 min centrifugation each time. The obtained pellet was mixed in 20 µL of PBS, applied onto regular microscope slides and mounted with PBS under coverslips. Fluorescent images were obtained with a Zeiss Axioplan II microscope (Zeiss, Oberkochen, Germany) equipped with Filterblock II (09), 450-490 nm, FT 510, LP 520 and a Plan-ApoChromat 100×/1.4 oil objective. A Zeiss AxioCam MRc digital camera run by Zeiss AxioVision 4 software was used and micrographs were taken with the Axiocam software (Zeiss) (31). The acquired raw images were adjusted and amended with Photoshop CS6 program (Adobe Creative, San Jose, CA, USA) for improving the qualitative aspects, such as brightness, contrast, and reducing background signals (32).

MALDI-TOF MS

MALDI-TOF MS (Bruker Daltonics) was used to analyze the mass range of peptides and to investigate differences between hLF(1-11) and FITC-hLF(1-11) based peaks. Accordingly, different concentrations of hLF(1-11) ranging between 1, 0.1, and 0.01 mg mL^{-1} and FITC-hLF(1-11) ranging between 1, 0.5, and 0.05 mg mL^{-1} were prepared in 1× PBS pH 7.4. To identify *Candida* and bacterial species, full protein extraction was performed using fresh overnight cultures from solid media. Protein extraction was done according to the MALDI Biotyper standard protocol as described elsewhere (33). Briefly, a colony of the freshly cultured microorganisms was added to a volume of 1,200 µl ethanol 70%, and the suspension was centrifuged. The supernatant was discarded, and according to the size of the pellet, equal amounts of formic acid 70% (Merck, Darmstadt, Germany) and acetonitrile 100% (Roth, Karlsruhe, Germany) were added to the pellet. After centrifugation, 1 µL of supernatant was pipetted in duplicate onto a polished steel target plate (Bruker Daltonik) and overlaid with 1 µL of hLF(1-11) or FITC-hLF(1-11). Two different concentrations were used of each peptide, i.e. 0.1 and 0.01 mg mL^{-1} for hLF(1-11) and 0.5 and 0.05 mg mL^{-1} for FITC-hLF(1-11), respectively. After air-drying, 1 µL of MALDI matrix (10 mg ml^{-1} of α-cyano-4-hydroxy-cinnamic acid [α-HCCA] in 50% acetonitrile–2.5% trifluoroacetic acid; Bruker Daltonik) was added onto all spots. MALDI-TOF MS spectra were acquired with a Microflex LT/SH mass spectrometer (Bruker Daltonics) calibrated with a bacterial test standard (BTS; Bruker Daltonik) in the mass range 2–20 kDa (34). Acquired spectra by MALDI-TOF MS were analyzed by Flex Analysis prototype software v3.3 and identification was performed by MALDI Biotyper OC v3.1 (all Bruker Daltonik).

Results

***In vitro* binding of ^{99m}Tc-hLF(1-11) to *Candida* and to blood cells**

Experiments with technetium labeled peptides were used to study binding of the peptide to *Candida* cells and if they were comparable with the results found using FITC-hLF(1-11). Counting radioactivity in cell fractions revealed that ^{99m}Tc-hLF(1-11) binds at a significantly higher rate (p<0.01) to *C. albicans* in blood than with blood cells alone. Results indicated that in whole blood, the peptides were found in 87.8 ± 0.5% of the plasma fraction for all three concentrations of hLF1-11 followed with binding of 5.5 ± 0.6% to erythrocytes, 3.9 ± 0.4% to thrombocytes and 2.8 ± 0.3% to leukocytes (Table 1). Experiments with a mixture of ^{99m}Tc-hLF(1-11) and *Candida* cells in whole blood showed that after 1 h of incubation, 76,7% of the labeled peptides were bound to the *Candida* cells and 13,5% leukocytes, and 9,7% to erythrocytes. These results indicated that ^{99m}Tc-hLF(1-11) has a high affinity to bind to *Candida* cells when compared to human blood cells.

Table 1. *In vitro* binding assay and distribution of ^{99m}Tc-hLF(1-11) fraction counts of whole blood samples. Radioactive-labelled peptides were found for 87.8% in the plasma fraction, for 5.4% to erythrocytes, 3.9% to thrombocytes and 2.8% to leukocytes as calculated after radioactivity counting (3×) in fractions obtained after layered Ficoll density centrifugation. An average and standard deviation for three countings were calculated.

Plasma (%)	Erythrocytes (%)	Thrombocytes (%)	Leukocytes (%)
88.1	4.8	4	3
87.2	5.6	4.2	3
88.1	5.9	3.4	2.5
87.8 ± 0.5	5.5 ± 0.6	3.9 ± 0.4	2.8 ± 0.3

Fluorescence microscopy

Sensitivity of binding of FITC-hLF(1-11) to the *Candida* and bacterial cells was evaluated using yeast and bacterial isolates growing on agar plates. The results showed that ≥98% *C. albicans* stained after 15 min incubation time (Figure 1a). The same results were obtained for *C. auris*, *C. glabrata*, *C. parapsilosis* and *C. tropicalis*. FITC-hLF(1-11) also bind to the bacterial cells within 15 min incubation time and stained the entire cells (Figure 1c). As an example, Figures 2a and 2c show the results of FITC-hLF(1-11) stained *C. albicans* and *S. aureus* derived from GYPA and TSA plates, respectively.

To investigate whether FITC-hLF(1-11) binds to human blood cells, whole blood samples containing FITC-hLF(1-11) were analyzed by fluorescence microscopy. The results illustrated that there was no binding between FITC-hLF(1-11) and human blood cells (Figure 1b). The next evaluation was to detect reference and clinical *Candida* and bacterial species from spiked blood samples after lysis of blood cells. Cells of all *Candida* and bacterial isolates stained with FITC-hLF(1-11), and the percentage of labeled cells was ≥95% for the *Candida* isolates and ~80% for the bacterial isolates according to results from cell counts (data not shown). The cells of the various *Candida* species differed in morphology and size, but most of them were uniformly labeled. The same results were obtained for both reference and clinical *Candida* isolates growing on agar plates and in spiked blood samples (Figure 1a, 1b). Gram-positive and Gram-negative bacteria were used to evaluate whether binding FITC-hLF(1-11) is specific to *Candida* cells or may bind to other microorganisms that also may occur in BSI. The results showed that bacterial cells were also labeled (Figure 1c). This observation implies a broader application of this peptide for the detection of a broader range of microorganisms.

Figure 1a.

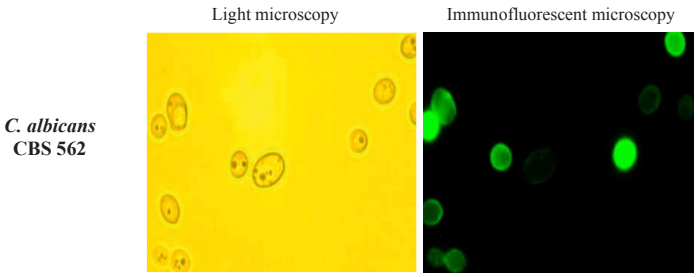
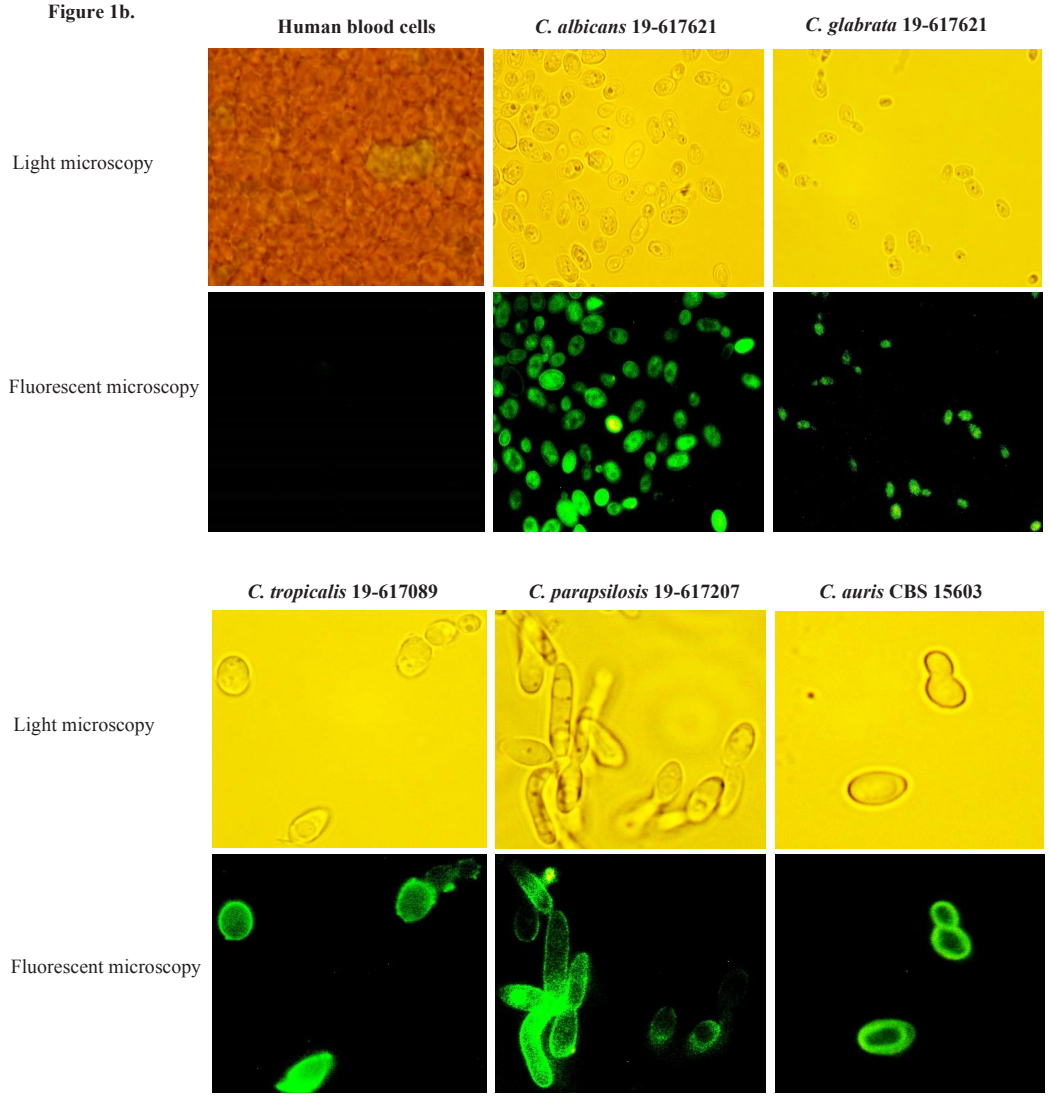


Figure 1b.



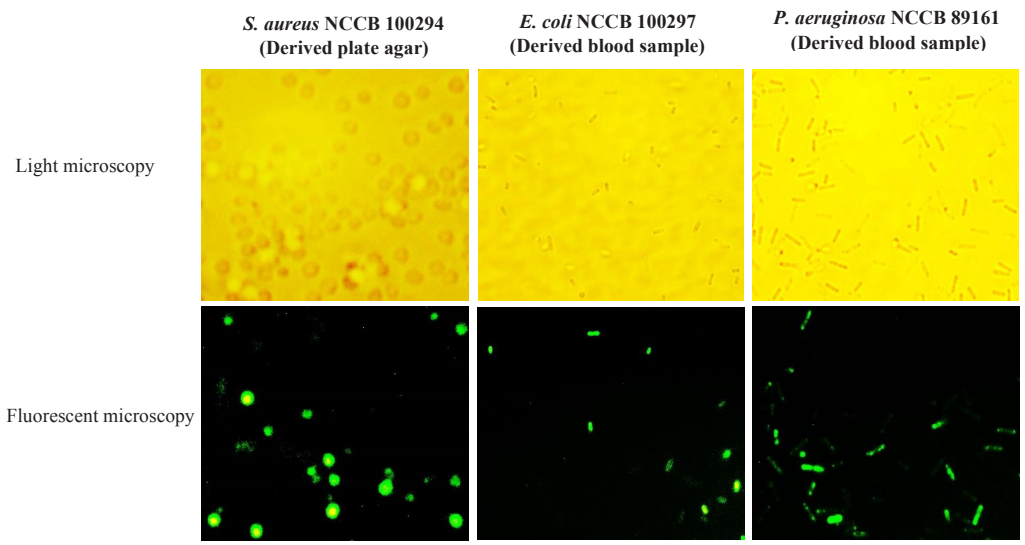


Figure 1. Binding of FITC-hLF(1-11) peptide to *Candida* and bacterial cells. (a) *Candida albicans* derived from plate agar were labeled by FITC-hLF(1-11). The results showed that all *Candida* species were stained. (b) Human blood cells were incubated with FITC-hLF(1-11) to test the affinity of this peptide to human cells. The results indicated that there is no binding between FITC-hLF(1-11) and human blood cells. Five clinically relevant *Candida* species were correctly identified after extracting from spiked broth blood cultures labeled with FITC-hLF(1-11). (c) *S. aureus* derived from plate agar and *E. coli* and *P. aeruginosa* derived from spiked broth blood cultures were also labeled with FITC-hLF(1-11).

Species identification by MALDI-TOF MS

Analysis in PBS. Using MALDI-TOF MS the hLF(1-11) peaks occurred in a range between 2700 and 8000 m/z. At three different concentrations (1, 0.1, and 0.01 mg mL^{-1}), hLF(1-11) showed a predominant peak with a size of about 2850 m/z. Two other smaller peaks were observed at a hLF(1-11) concentration of 1 mg mL^{-1}, but with lower intensity. These two small peaks were at about 10^3 a.u. (arbitrary unit) intensity at concentrations of 0.1 mg mL^{-1} and 0.01 mg mL^{-1} compared to 15×10^3 a.u. intensity at concentrations of 1 mg mL^{-1} (Figure 2a). FITC-hLF(1-11) peaks occurred in a range between 2700–6000 m/z at concentrations of 1, 0.5 and 0.05 mg mL^{-1}. The same peak with a size of 2850 m/z detected in hLF(1-11) was also observed in FITC-hLF(1-11). Additionally, two other main peaks at a range of 3000 m/z with an intensity about 10^4 a.u. were detected for this peptide that was not observed in hLF(1-11). These new peaks might appear due to the labeled probe bound to hLF(1-11) (Figure 2a).

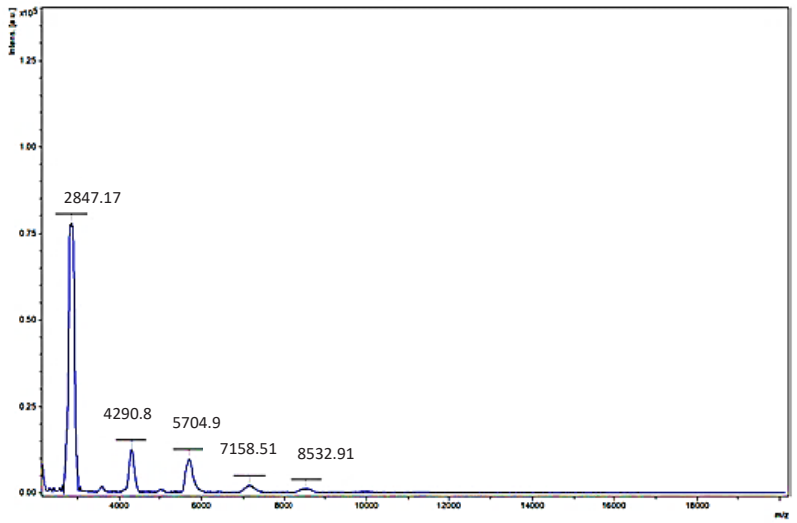
Identification of isolates derived from plate agar. Five main reference *Candida* and three bacterial species were identified by MALDI-TOF MS after binding to three concentrations of hLF(1-11) and FITC-hLF(1-11). The results showed that at higher concentrations of hLF(1-11) (1 and 0.1 mg mL^{-1}) and FITC-hLF(1-11) (1 and 0.5 mg mL^{-1}) peaks of *Candida* and the bacterial species were suppressed and identification was not possible. Therefore, to identify the reference and clinical isolates, concentrations of 0.01 and 0.05 mg mL^{-1} were used for hLF(1-11) and FITC-hLF(1-11), respectively. All reference *Candida* and bacterial isolates enriched with these peptides using the defined concentrations were correctly identified by MALDI-TOF MS. As an example, *C. albicans* CBS 562 and *E. coli* NCCB 100297 are shown in Figure 2b. Adjusting peptide concentrations created a balance for the peak's intensity between peptides

and *C. albicans* and *E. coli* that resulting in the successful identification of both isolates with high log (score) values ≥2.

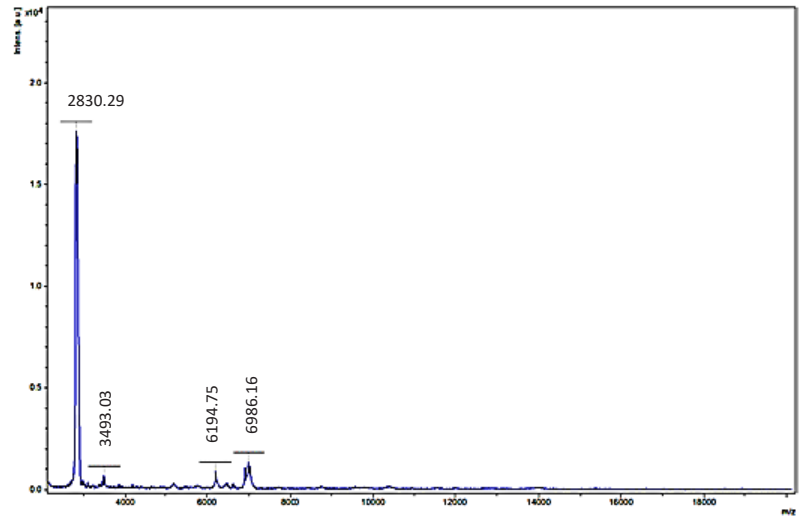
Identification of *Candida* clinical isolates derived spiked blood sample. Identification using MALDI-TOF MS of different clinical isolates derived from spiked blood samples was performed based on yeast cells bound to hLF(1-11) and FITC-hLF(1-11). Some species, such as *C. auris*, *C. albicans*, and *C. glabrata* were successfully identified in the mass range of 2–20kDa (Figure 2 c). *C. glabrata* and *C. auris* were identified with log scores ≥1.7, but *C. albicans* had the highest log score ≥2. In contrast, *C. parapsilosis* and *C. tropicalis* could not be identified after binding with hLF(1-11) and FITC-hLF(1-11), even when the identification was repeated for 3 biological samples and 3 technical experiments. For instance, Figure 2c compared the number of presented peaks acquired by *C. albicans*, *C. glabrata*, and *C. parapsilosis* and their identification alone and along with peptides. The results showed that *C. parapsilosis* peaks were not presented along with hLF(1-11) and FITC-hLF(1-11) that led to a lack of identification of these species, while it was correctly identified alone. Contrarily, *C. albicans* and *C. glabrata* presented several peaks with high intensity that in combination with hLF(1-11) and FITC-hLF(1-11) peaks could be identified correctly resulting in an identification of those isolates.

Figure 2a. hLF(1-11) only

1 mg mL^{-1}

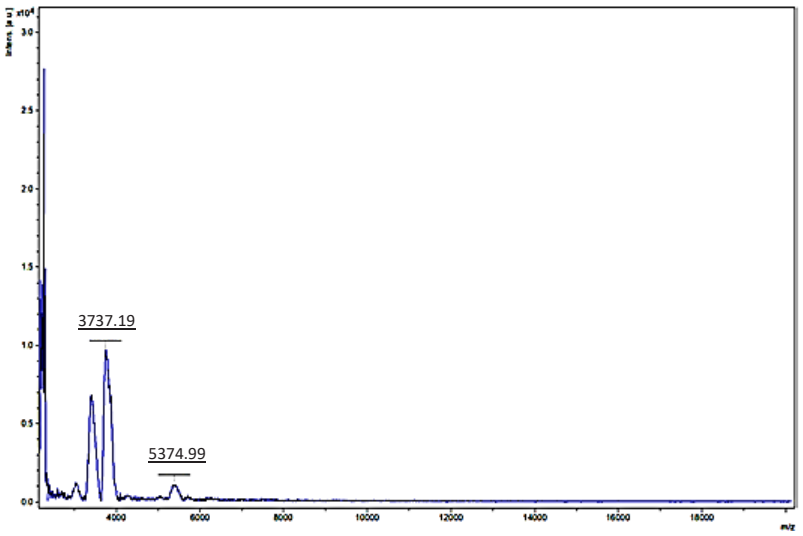


0.01 mg mL^{-1}



FITC-hLF(1-11) only

1 mg mL^{-1}



0.05 mg mL^{-1}

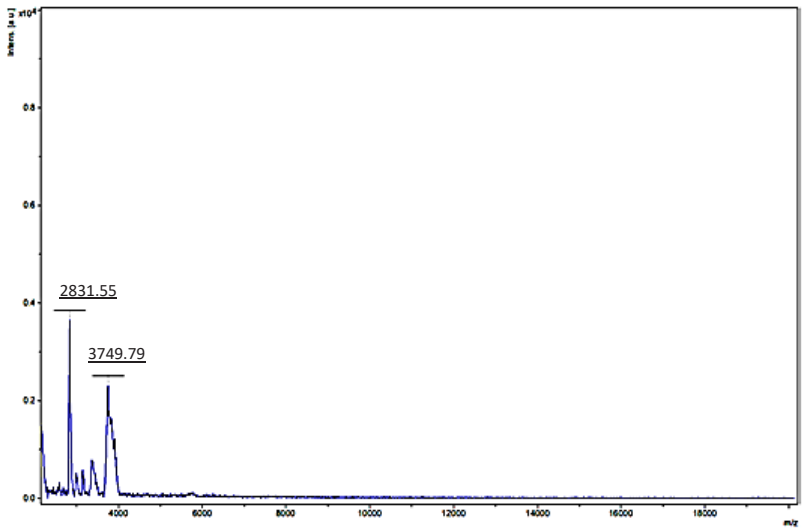
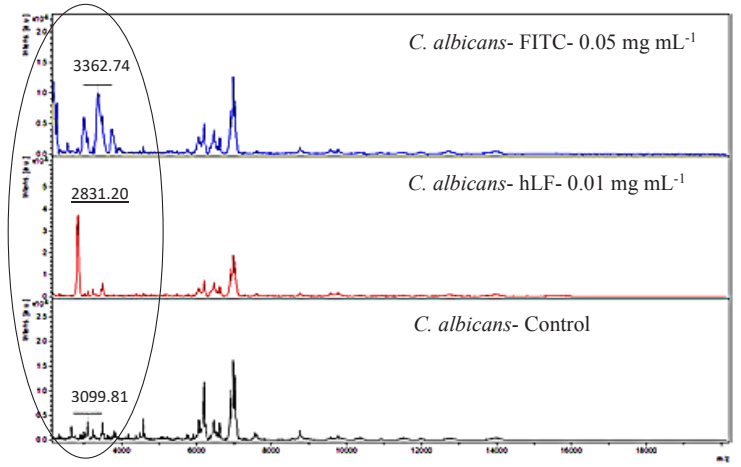


Figure 2b.

C. albicans
CBS 562



E. coli
NCCB 100297

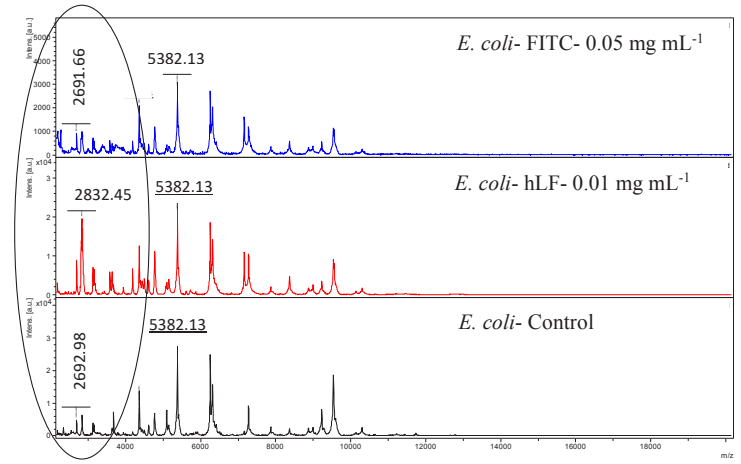
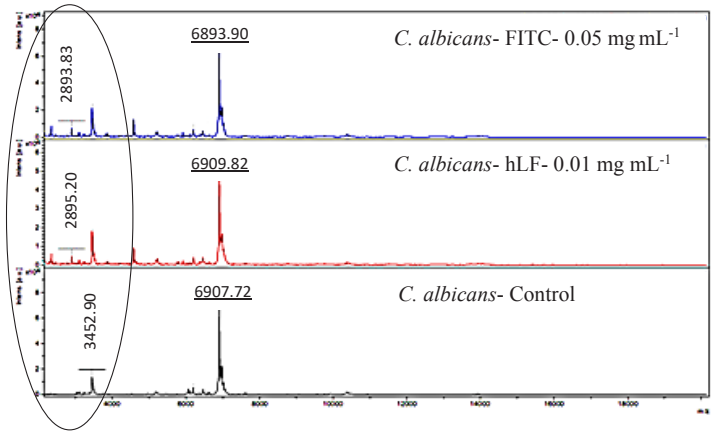
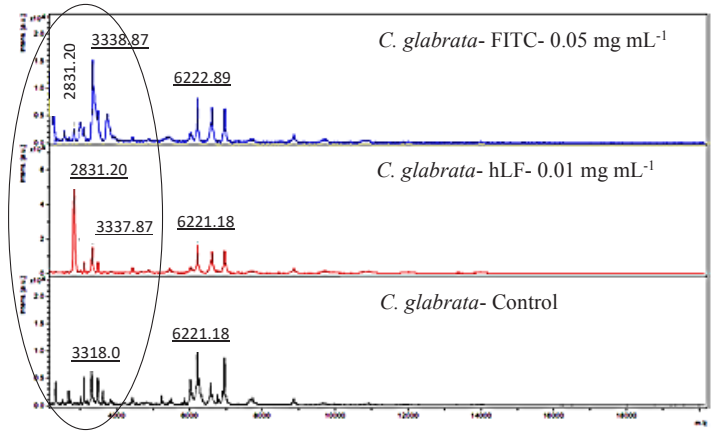


Figure 2c.

C. albicans
19-617621



C. glabrata
19-617621



C. parapsilosis
19-617207

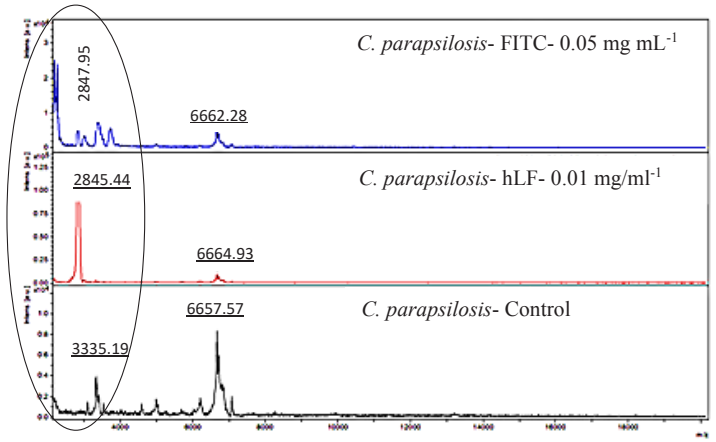


Figure 2. Identification with MALDI-TOF MS of hLF(1-11) and FITC-labeled hLF(1-11) peptide peaks alone and with *Candida* and bacterial isolates. (a) Defined hLF(1-11) and FITC-peptide peaks are shown at highest and lowest concentrations of 1 and 0.01 mg mL^{-1}, and 1 and 0.05 mg mL^{-1}, respectively. (b) Reference *C. albicans* isolate CBS 562 and *E. coli* NCCB 100297 alone and bound to hLF(1-11) concentration 0.01 mg mL^{-1} and FITC-hLF(1-11) concentration 0.05 mg mL^{-1} were correctly identified by MALDI-TOF MS with high log score ≥2. (c) Identification of clinically obtained *C. albicans*, *C. glabrata*, and *C. parapsilosis* bound to peptides from spiked blood samples. *C. albicans* had the highest number of detected peaks and along peptides could identify with high log score ≥ 2. *C. glabrata* showed lower numbers of peaks and intensity, but it could be identified with a log score ≥1.7. *C. parapsilosis* with a very low number of peaks was not identified, likely due to suppression of its peaks by hLF(1-11) and FITC-hLF(1-11).

Discussion

This study confirmed the possibility of hLF(1-11) as a new compound useful for the detection of *Candida* and bacterial cells in human blood samples. The number of *Candida* BSI has been growing dramatically and is globally characterized by high mortality values ranging between 23 to 50% (7). Hence, accurate and rapid methods to diagnose BSI caused by *Candida* species are urgently required, and even more importantly, a technique that can identify *Candida* species directly from blood samples is urgently needed (19, 35). Our current study shows a high binding capability of hLF(1-11) to *Candida* cells as evaluated by two approaches, namely labeling with ^{99m}Tc-hLF(1-11) and by FITC-peptides, that may be interesting in this respect. Our findings confirmed the binding of the peptides to the *Candida* cells which show low affinity to human cells, thus suggesting the applicability of hLF(1-11) for the detection and identification of microorganisms directly from blood samples without interference with human cells. This result was supported by results obtained by an *in vitro* ^{99m}Tc-hLF(1-11) and fluorescence microscopy observations using FITC-labeled peptides. Additionally, our findings supported previously made observations by Welling *et al.* (2002) conducted with bacteria (30). They tested sensitivity a technetium-99m-labeled peptide derived from ubiquicidine binding to *Staphylococcus aureus in vitro*, and later, in *S. aureus*-infected mice. They observed a high affinity of ^{99m}Tc-peptide to *S. aureus in vitro*. Furthermore, injection of this peptide into the infected-mice showed a higher accumulation of labeled-peptide at the site of infection that resulted in a reduction of infection. Importantly, the affinity of ^{99m}Tc-peptide to mice blood cells was less than for *S. aureus* cells (30).

Recently, synthetic peptides gained interest to be used for the identification of pathogenic microorganism (19, 36). Among synthetic peptides, hLF(1-11) with two cationic domains (37), is known for its anti-inflammatory and antifungal activities (26–28, 38–40). Apparently, this cationic peptide binds within seconds to *Candida* cell walls due as they are negatively charged,

and after accumulation, they penetrate into the cells. The way of penetration is not yet clear, but it may be similar to other peptides that use the direct penetration mechanism to enter the cells via the membrane bilayer, followed by induction of fusion between membranes without any associated leakage event, or, alternatively, they may penetrate cells via an energy-dependent endocytosis process (26, 38, 41–43). The short time period that the peptides are located at the cell wall is the proper time to be used for the detection and identification of the respective microorganisms. In this study, the new applicability of the antimicrobial peptide hLF(1-11) and its derivatives to identify *Candida* species from agar cultures and directly from spiked blood samples, using a short incubation time, was investigated. The results showed fast and high binding capacity to these yeasts in contrast to the human blood cells. Similar results were obtained for bacterial cells, thus clearly showing that this peptide is not specific for *Candida* species.

Despite the rapid development of molecular-based identification technologies, they are not yet widely accepted and too expensive for use in all clinical laboratories (44, 45). Here, we explored the identification of pathogenic yeasts bound to peptides using MALDI-TOF MS. Both hLF(1-11) and FITC-hLF(1-11) presented peaks in the range of 2–20 kDa that makes them detectable by MALDI-TOF MS. However, the limitation of MALDI-TOF MS is that this method needs a cell density between 2.40×10^{8} and 1.10×10^{10} viable cell counts (VCCs) per mL (46), while in the current study we tried to minimalize the identification to up to 1000 cells mL^{-1}. In future clinical applications, the detection of even 1-10 cells mL^{-1} needs to be explored. A combination of rapid and accurate extraction of proteins from 1–10 cells ml^{-1} of sample with hLF(1-11) and labeling with FITC-hLF(1-11) might overcome this issue. In our study, identification of three bacterial species as reference isolates and the clinically most important *Candida* species, viz., *C. auris*, *C. albicans*, and *C. glabrata*, both reference and clinical isolates, derived from agar cultures and spiked blood samples with 1,000 cell mL^{-1} enriched

with two different peptides was successfully performed by MALDI-TOF MS. However, further analysis is required to solve the drawbacks observed for some clinical *C. parapsilosis* and *C. tropicalis* isolates derived from spiked blood samples that could not be identified. Thus, to detect at least 10 cells of microorganisms in 1 ml of blood sample the protein extraction method needs further improvement and adjusting the peptide concentrations to the intensity of extracted protein to avoid suppression of microorganism-based peaks by the peptide. Further optimization of MALDI-TOF MS by improving different parameters of the Microflex LT/SH mass spectrometer software (Bruker Daltonics), such as laser power, number of laser shots per spot, peaks intensity, mass range, and so on, is required in order to detect peaks derived from fewer cells in blood samples and to discriminate between them and those from background signal noises.

The hLF(1-11)-labeled microbial cells can also be used to detect live microorganisms in a flow-based system. As already shown in some studies, peptides alone or along with FITC-hLF(1-11) can be conjugated to different cells, such as cancer or immune cells, that then can be detected by flow cytometry (47–49). Therefore, the detection of lower numbers of *Candida* cells labeled with hLF(1-11) could be improved using flow cytometry. Accordingly, FITC-hLF(1-11) can also be bound to a specific antibody generated against a particular antigen of the respective pathogen in order to improve the sensitivity and specificity of detection. Additionally, flow cytometry allows to detect not only single but also multiple pathogenic microbes in a single clinical sample using specific fluorescent probes, such as antibodies and RNA probes. Therefore, the combination of FITC-hLF(1-11) with specific probes could increase the sensitivity of this method to detect multiple bacteria/fungi involved in BSI (50, 51). This method, however, is rather expensive, complicated to use, and not validated yet to be applied in routine clinical laboratories (52, 53).

Another possibility is Nanowire sensor technology that has been developed in the last decade (54–56). Newly developed biosensors exploring new applications are becoming more and more important for the analysis of protein biomarkers and as a diagnostic tool in the clinic. The current use of biosensors in microbial diagnostics is based on various technologies, such as immunosorbent assay (ELISA), amplification of a sample by polymerase chain reaction (PCR), and other detection methods like gel electrophoresis (57, 58). Unfortunately, these methods are costly and need long sample preparation and handling times. In addition, incorrect identifications may occur due to cross-reactions between enzymes and antibodies, low sensitivity of primers to the target, and false-positive PCR results due to contamination of extracted DNA. Biosensors relying on nanotechnological platforms might overcome these disadvantages due to their high sensitivity, rapidness, and cost-effectiveness (57, 58).

In summary, a new application of hLF(1-11)-labeled *Candida* cells showed new possibilities for the enrichment, detection and identification of low numbers of pathogenic yeast cells in blood samples. This proof of concept study showed that the peptides may indeed be useful for that purpose, but to reach the final goal to detect as low as about 10 cells of microorganisms in 1 ml of blood sample, many efforts and investigations are still needed. Finding a similar, average incubation time for all microorganisms involved in BSI in the presence of the determined peptide concentration in order to obtain maximum binding to the microbe cell walls with having minimum labeling of the peptide inside the cells is important in this respect. Besides, using a method that could detect and identify fewer numbers of cells bound to the peptides in blood samples is crucial (19, 36) and biosensor technology may be useful in this respect.

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Chapter 8

General discussion

Invasive candidiasis caused by prevalent and newly emerging *Candida* species is increasing due to the expanding number of immunocompromised or otherwise vulnerable patients. Next to this trend the availability of only a limited number of antifungals and increasing resistance of *Candida* isolates to some of those drugs is worrying (1, 2, 3). To choose the right antifungal therapy, accurate identification of *Candida* isolates at the species level is required and this implicates precise and rapid diagnosis methods (4, 5). Besides the ongoing efforts to develop and improve diagnostic methods, the discovery of new antifungal drug targets, and accordingly, the development of new antifungals is also in progress (6). Additionally, antifungal susceptibility testing using conventional methods for *Candida* species is time-consuming, and molecular methods are costly and not commonly used in routine clinical laboratories. Therefore, a rapid, accurate, and affordable method for testing susceptibility is required that is informative to make the correct treatment decisions. Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) is widely used in routine clinical laboratories for the rapid identification of bacteria and fungal isolates. This method is accurate, rapid, and does not need extensive training before use. Therefore, expanding its application to the antibiotic/antifungal susceptibility testing (AST/AFST) has been considered (7, 8).

Invasive candidiasis is a serious and lethal infection, and the causative agents need to be identified and treated as fast as possible. Mortality rates of *Candida* bloodstream infections (BSI) vary between 40-50%, depending on the geographical distribution, the species involved and the patient population. Delay in appropriate therapy increases the risk of mortality (9, 10). To reduce the time of detection of a resistant isolate is the most important reason to develop a novel, fast and reliable AST/AFST method. Accordingly, significant efforts have been focused

on the development of new technologies that shorten the time to generate results (11, 12). The development of a rapid, accurate AFST method is challenging. It has led to the development of several commercial and non-commercial methods. Some of them are already introduced in routine clinical laboratories, but most of them are culture-based approaches. The widely used standard microdilution methods, i.e. the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methods, the older commercial approaches, i.e. disc diffusion and Etest, and the new colorimetric microdilution test (Sensititre® YeastOne) all need at least 72h from the time the blood sample infected with microbes is taken to release the final results to the clinician.

The MicroScan WalkAway (Beckman Coulter) is one of the automated systems used for bacterial identification and susceptibility testing. This method is a rapid and conventional technology with minimal maintenance. The MicroScan WalkAway is a photometer or fluorometer system to determine growth development. It has been constructed to perform susceptibility test in 3.5–7 h for Gram-negative, and 4.5–18 h for Gram-positive bacteria. However, it has not yet been applied to fungi (13-15). In this regard, the BD Phoenix Automated Microbiology System (BD Diagnostics) is another automated identification and susceptibility testing system based on both turbidometric and colorimetric (oxidation-reduction indicator) growth detection. This is an accurate, rapid and reliable method used for the detection of known and emerging antimicrobial resistance. It can release data between 6 and 16 h for Gram-negative and Gram-positive bacteria. This technology has been mostly applied to test β-lactam susceptibility of *Pseudomonas aeruginosa*, and detection of carbapenem resistance in *Klebsiella pneumoniae* against meropenem and imipenem. This method decreases the time to obtain the results, but it still takes longer than a working day to investigate some bacteria species for various antibiotics. Additionally, it has not yet been developed to perform AFST for fungi (16-18).

The Vitek 2 system (BioMérieux) is the only available fully automated method used for bacterial and fungi identification as well as antibiotic/antifungal susceptibility testing. The Vitek 2 system is a fluorescence-based technology and performs a susceptibility test with an average time of 6-17h for different bacteria and 8-15h for yeasts (19, 20). However, a 15h period to obtain results is still a long time for patients who are threatened to die from invasive candidiasis. Furthermore, a 15h detection time requires two groups of technicians from the same laboratory working on one clinical sample which is considered a drawback in the clinic. Therefore, a novel rapid method that can detect resistant bacteria and *Candida* isolates with the potential for automation in less than one working day is urgently required.

Hence, MALDI Biotyper antibiotic/antifungal susceptibility test rapid assay (MBT ASTRA) was developed by Bruker company (Bruker Daltonics GmbH, Bremen, Germany). MBT ASTRA is a semi-quantitative assay based on the growth of microbial cells in a control compared to growth in the presence of different concentrations of antibiotic/antifungal compounds. This semi-automated method is based on MALDI-TOF MS and has the ability to release results at an average time between 3-6 h for bacteria (21-23). MBT ASTRA is still under development and has not yet been released for use in routine clinical laboratories, but it is ready for use in research laboratories. So far, it is the most rapid available method for AST with high reproducibility and accuracy. Furthermore, this approach is cost-effective if the MALDI-TOF MS equipment is already present in the laboratory. The main body of this Ph.D. project was to develop and optimize this method to investigate its use for AFST of yeasts to echinocandins in a short turnaround time of 7h (24-26).

In chapter 1, we demonstrated general information about yeast species, focusing on *Candida albicans*, *Candida glabrata*, and *Candida auris* that are used in this thesis. Available antifungals, their function, and reasons of the growing number of *Candida* isolates resistant to these antifungals are described. Next, methods to detect resistant *Candida* isolates in order to

have proper patient management are discussed. Afterwards, conventional and new rapid diagnostic methods are described and new trends for the diagnosis pathogenic microorganisms in clinical laboratories discussed. MALDI-TOF MS is described in detail as a multifunctional method used for diagnosis, AST/AFST, and typing assays.

In chapter 2, we describe the optimization of the MBT ASTRA as a novel, rapid method for AFST for microbial pathogens. We demonstrated the usefulness of MBT ASTRA, its advantages and disadvantages, and all aspects that needed to be considered to set up this method. For instance, sample preparation from Sabouraud dextrose agar (SDA) and spiked blood samples, blood lysis buffers, incubation times, protein extraction methods, introduction of the MBT ASTRA standard II, and set up the MS ASTRA prototype to analyze data are described in detail. As a result, we provide a protocol for using MBT ASTRA for AFST of *Candida* yeasts. An outstanding feature of this method is the short time required to perform the experiment which takes only 7h from obtaining the blood culture to get the susceptibility values. This means that in less than one working day MBT ASTRA can detect echinocandin resistant isolates of *C. albicans*, *C. glabrata*, and *C. auris*. So far, this is the fastest method for AFST for yeasts, but at present, it can only be used for research purposes. We, however, foresee that it will be used in routine clinical laboratories soon. To reduce the workload in routine clinical laboratories, fully automated instruments and technologies are strongly desired. Therefore, the hands-on work of MBT ASTRA needs to be minimized by automation before it will be introduced into routine clinical laboratory practice. Furthermore, expanding the application of this method to other *Candida* and *Aspergillus* species for AFST of echinocandins as well as other antifungals, with a priority to azoles, is also essential. Up to now, MBT ASTRA can detect susceptible and resistant *Candida* isolates against echinocandins, but intermediate isolates cannot yet be detected, and these are presently considered as resistant. In antifungal stewardship therapy, however, intermediate isolates are also categorized as resistant, and,

therefore, this is not a serious drawback since the intermediate isolates are detected as resistant by MBT ASTRA. However, misclassification of a resistant isolate as a susceptible reduces the sensitivity of this method and may result in a wrong therapy that leads to an increase of patient morbidity and mortality. Therefore, further optimization would be useful to detect accurately intermediate strains.

In chapter 3 optimization and use of MBT ASTRA was described for two *Candida* species, namely *C. albicans* and *C. glabrata*, against echinocandins using cultures growing on SDA. The observed high sensitivity and specificity showed the high potential of this method for use to test susceptibility to echinocandins of *Candida* spp. with a short incubation time (25). Unfortunately, MBT ASTRA could not detect intermediate isolates in this study, and those strains were considered as resistant. We did not observe any *C. albicans* intermediate strains in our sample, but for *C. glabrata* 19 strains had a minimal inhibitory concentrations (MIC) of 0.25 µg/ml and these were classified as intermediate. Only one out of 19 intermediate isolates was wrongly detected as a susceptible isolate by MBT ASTRA. Additionally, the paradoxical effect was observed for *C. albicans* isolates using a higher concentration of caspofungin. Although caspofungin is commonly used for *in vivo* treatment of patients without proof of the paradoxical phenomenon, it is not recommended to be used *in vitro* AFST to investigate the susceptibility of the yeast cells for this compound. A higher concentration of caspofungin likely causes stress to *C. albicans* isolates and leads to overproduction of chitin in the cell wall. This phenomenon increases the MIC and tolerance to antifungals by changing the cell wall construction due to raising the expression of the chitin biosynthetic pathways that, apparently, is not related to a mutation in *FKS1* and *FKS2* genes (27). Therefore, **in chapter 4** MBT ASTRA was further evaluated for testing anidulafungin susceptibility of *C. glabrata* isolates. We moved one step further by assessing the applicability of this method to detect resistant *Candida* isolates that were directly obtained from spiked broth blood cultures. The MBT

ASTRA method showed great promise for AFST of *C. glabrata* isolates directly obtained from broth blood cultures. In this study, the susceptibility of 100 *C. glabrata* isolates was tested by three different approaches, namely the CLSI method, sequencing of hotspot 1 and hotspot 2 regions of the *FKS1* and *FKS2* genes, and MBT ASTRA. The acquired results by MBT ASTRA were compared with those obtained with the two other approaches, one of them phenotypic and the other a genotypic assay. When compared with the molecular sequence approach, MBT ASTRA has a sensitivity and specificity of 100% and 80%, respectively. When compared to microdilution, MBT ASTRA showed a sensitivity and specificity of 80% and 95%, respectively. Within 7h, MBT ASTRA showed promising results with high sensitivity and specificity. The only challenging outcome was the lower sensitivity of MBT ASTRA when compared to the CLSI method that resulted in misclassification of some intermediate *C. glabrata* isolates that were classified as susceptible. One of the reasons to explain this result relates to the method used for lysis of the spiked blood samples. In this study, the MALDI Sepsityper kit (Bruker Daltonik GmbH, Germany) was used and the lysis buffer and several washing steps may change the cell wall structure that may impact the interaction of anidulafungin with its target. Therefore, it is recommended to try other methods to optimize lysis of the blood cells that may have less effect on the cell wall structure of the yeasts. Another effort should focus on the improvement of the MBT ASTRA method and the MS ASTRA prototype software (Bruker Daltonik) to allow correct detection of intermediate isolates. To reach this aim, using a larger sample size, varying antifungal and MBT ASTRA standard II concentrations, using different incubation times, and any other possible variations of the MBT ASTRA procedure, as well as manipulation and improvement of the MS ASTRA software should be investigated.

Since this method was developed for application in routine clinical laboratories, it was decided to evaluate the application of MBT ASTRA directly using patient's blood samples. Therefore,

MBT ASTRA was tested for two weeks in a single routine clinical laboratory. In total, six blood samples infected by *C. glabrata* could be collected, and all were first tested using the disc diffusion to test susceptibility for anidulafungin. The results showed that the isolates were all susceptible to anidulafungin after overnight incubation. Using MBT ASTRA with a shorter incubation time of 6h, the results could be released within 7h. Five out of six *C. glabrata* susceptible isolates were correctly detected as susceptible and one isolate was categorized as resistant (see figure 6 in Chapter 2). Since the disc diffusion is not the gold standard method for the susceptibility testing of yeasts (28), it is possible that the results acquired by MBT ASTRA were also correct for that one strain. One of the drawbacks related to the disc diffusion method is trailing growth in which reduced, but persistent growth of *Candida* species occurs at high concentrations of antifungals. The mechanisms of trailing growth are not clearly understood, but several factors, like inoculum size, incubation time, medium pH, and buffer concentration may contribute to this phenomenon (29). However, further analysis including a comparison with the reference microdilution methods, i.e. the CLSI and EUCAST methods, and/or sequence analysis of *FKS1* and *FKS2* genes is required to evaluate and compare the results correctly. Due to the time limitation of two weeks needed to set up MBT ASTRA in a routine clinical laboratory, it was not possible to perform further analysis by phenotyping and genotyping assays, but those six patient's samples are still available to test with reference susceptibility testing methods. Overall, MBT ASTRA showed a promising performance using cultures from SDA and the direct use of clinical materials, such as infected blood, in routine clinical laboratories. However, according to the time-limitation in routine clinical laboratories, hands-on-work of this method is still more than laboratory technicians desire. Therefore, automization of this method is required before introducing MBT ASTRA to the market.

In **chapter 5** MBT ASTRA was used to investigate *C. auris* as a multidrug-resistant *Candida* species. Since echinocandins are used as an empirical available antifungal against *C. auris,*

emerging resistant isolates to echinocandins are of risk for patients in hospitals. Therefore, fast detection of resistant *C. auris* isolates to echinocandins is important. MBT ASTRA was used to test the susceptibility of *C. auris* isolates derived from both SDA and broth blood culture against anidulafungin, micafungin and caspofungin. The results were very convincing as a cut off value against all three antifungals could be set. The same setup was performed for *C. auris* against the respective antifungals using the CLSI reference method. Standard microdilution methods have not yet determined the MICs for all available antifungals for *C. auris*. Therefore, we relied on the MICs acquired by the CLSI method in our experiments for this comparison. Caspofungin was excluded as the MICs cannot be reliably assessed using microdilution methods (see above), but a high agreement was obtained between the results obtained with the two methods for anidulafungin and micafungin AFST of *C. auris*. Thus, our data indicated that MBT ASTRA is also applicable for AFST of newly emerging *Candida* species (24).

Owing to the importance of *C. auris* as a nosocomial infection that spreads rapidly and causes hospital outbreaks at a global scale, it is also crucial to find a way to detect *C. auris* outbreaks in hospitals. Accordingly, **in chapter 6**, the IR Biotyper was used for the first time to study the population characteristics of a set of *C. auris* isolates collected worldwide. The IR Biotyper was developed to study hospital outbreaks caused by different microorganisms, but so far the method has been applied mainly to bacteria. This technology has a rapid and convenient workflow that makes it useful to identify the source of hospital outbreaks in order to take proper measures to prevent their expansion (30). Hence, we evaluated the performance of IR Biotyper using different isolates of *C. auris*.

The acquired results by the IR Biotyper were compared with those obtained by MALDI-TOF MS as a protein-based method and other already established molecular typing methods, like microsatellite typing, ITS sequence analysis, and AFLP fingerprinting. The microsatellite typing assay provided 4 main clusters, MS I-IV, and was used as the reference standard typing

method in this study as it showed best concordance with data previously acquired by WGS. The IR Biotyper created two major clusters, namely IR I with 78 isolates and IR II with 18 isolates, respectively. The IR Biotyper cluster I was also divided into four subclusters (IR Ia-Id) and each of them contained isolates from the four main MS clusters. For instance, IR Ia, IR Ic, and IR Id included isolates from South Africa (= cluster MS III), South America (= cluster MS IV), and South Asia (= cluster MS I), respectively. Subcluster IR Ib included 11 isolates and five of them came from East Asia and the remaining isolates from South Africa and South Asia (= MS I, MS III). In contrast, due to the high level of diversity within genotype IR II, no subclusters were recognized. Cluster IR II included isolates from Belgium (from a Kuwaiti patient), India, Kuwait, and Malaysia (= MS I), and isolates from Spain (= MS III). Comparison of data obtained by IR Biotyper did not fully support the results from the microsatellite assay, and the co-phenetic correlation value between these two methods showed only a score of 0.33. In general, concordance between methods was low with the highest co-phenetic value of 0.47 being observed between ITS sequencing and microsatellite analysis. Comparison of the data acquired by IR Biotyper with MALDI-TOF MS, ITS sequencing, and AFLP resulted in even a poorer agreement between these methods. In general, typing data from five different molecular and biochemical typing methods did not result in a concordance rate above 50% between all methods. Therefore, to determine a reliable epidemiological map of *C. auris* during outbreaks and evaluate the reliability application of these typing methods, analysis of more isolates with a known clinical origin using WGS as reference and using a blinded approach is recommended. This obviously implies a lot of technical work and budget. In summary, the applicability of IR Biotyper as a biochemical typing method to investigate nosocomial outbreaks caused by *Candida* isolates requires further optimization in order to improve the resolution of this method. Moreover, further analysis by expanding the number of isolates to be tested and using

different *Candida* species could facilitate the final optimization of this method, and its subsequent application in medical mycology laboratories.

In **chapter 7**, the application of MALDI-TOF MS to identify pathogenic *Candida* cells labeled with a synthetic antimicrobial human lactoferrin protein hLF(1-11) peptide, an 11 amino acid peptide derived from the N-terminus of hLF was evaluated using blood samples. The antibiotic/antifungal activities of hLF(1-11) have already been investigated (31, 32). We evaluated for the first time the binding of labeled-hLF(1-11) peptide to the cell walls of *Candida* species in order to identify labeled cells directly derived from blood samples. The hLF(1-11) was firstly labeled with radioactive ^{99m}Tc and fluorescein isothiocyanate (FITC) to appraise the sensitivity of this peptide to bind to the *Candida* cells in whole blood. The binding assay was performed by measuring the radioactivity of ^{99m}Tc-hLF(1-11) using a VDC 101 dose calibrator, and in addition using FITC-labeled hLF(1-11) with fluorescence microscopy. The results showed a high affinity of the hLF(1-11) to the *Candida* cells, and in contrast, the numbers of labeled human blood cells were negligible. This outcome showed the possible applicability of this peptide for the enrichment and detection of pathogenic *Candida* isolates from blood without interference of human blood cells. Accordingly, the identification of labeled *Candida* cells with hLF(1-11) was performed by MALDI-TOF MS. In this experiment, the five clinically most important *Candida* species were tested and among them, *C. albicans*, *C. glabrata*, and *C. auris* labeled to hLF(1-11) could be successfully identified directly from whole blood. Unfortunately, lack of identification was observed for *C. parapsilosis* and *C. tropicalis* and this needs further analysis to eliminate this drawback. So far, we could directly identify three *Candida* species labeled with hLF(1-11) in a concentration of 1000 cells/ml in blood samples. In the future, the aim should be to identify most of the pathogenic *Candida* species with a number of cells of about 1-10 cells/ml using enrichment and detection by this peptide and, subsequent, identification by MALDI-TOF MS. To catch this urgent clinically

need, additional optimization should focus on improvement of the protein extraction method, and assessing the balance between peptide concentration and extracted protein from a low number of cells to allow identification by MALDI-TOF MS. For the latter manipulating several factors, including improved measuring and better software analytic programs, is essential. Furthermore, expansion of the application of hLF(1-11) for the identification of other pathogenic microorganisms using other diagnostic methods, such as microsensors, is foreseen. With respect to the high demands of developing an accurate, rapid, and culture-free method to diagnose bloodstream infections (BSI) caused by *Candida* and bacterial species directly from the patient's blood samples, makes this method a promising approach in the future.

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Chapter 9

Summary

In summary, this Ph.D. project focused on the optimization of a new method for the rapid detection of *Candida* species that are resistant to antifungals aiming at future use in routine clinical laboratories. The present study provides the first comprehensive assessment of MALDI-TOF MS for AFST against echinocandins. MBT ASTRA, a semi-quantitative method based on MALDI TOF MS, was optimized to detect *Candida* spp. resistant to echinocandins within 7h. The findings of this study suggest that MBT ASTRA can not only be used for susceptibility testing of *C. albicans*, *C. glabrata*, and *C. auris* against echinocandins, but that it also can be used for testing isolates of other *Candida* species against different antifungals, like azoles. The most important limitation lies in the fact that the hands-on-work required is still more than ideal for daily use in routine clinical laboratories. Any new method must be rapid with broad applicability and availability. Furthermore, to generate high-quality data, allow standardization and preferably have a reasonable price are other advantages of MBT ASTRA. Accordingly, MBT ASTRA has the potential to get used for testing a broad range of microorganisms against different antibiotics and antifungals. This method can also be automated to become the most rapid AFST method for use in routine clinical laboratories. Furthermore, if a MALDI-TOF MS is available in the lab, the price to apply MBT ASTRA per sample is affordable. MBT ASTRA is still in the research phase, therefore, the price for this method to apply in routine clinical laboratory has not been yet finalized. In addition, a comparative study has been performed on different typing methods for *C. auris*, and possibilities have been explored for the use of a lactoferine peptide (hL1-11) for the enrichment and identification of *C. albicans*.

The findings of this study showed that MALDI-TOF MS is not only an accurate and outstanding method for the identification of microbes, but its application in AFST has also been

approved. Therefore, it may open a new window for a broader application of the MALDI-TOF MS technique to be used in research and clinical laboratories in the future.

Summary in Dutch / Samenvatting in het Nederlands

Dit Ph.D. project was gericht op de optimalisatie van een nieuwe methode voor de snelle detectie van *Candida* soorten die resistent zijn voor schimmelwerende middelen met het oog op toekomstige toepassing in de kliniek. In onze studie hebben we de toepassing van MALDI-TOF MS getest voor gevoeligheidstesten voor echinocandines. De resultaten van deze studie suggereren dat MBT ASTRA niet alleen bruikbaar is voor het testen van de gevoeligheid voor echinocandines van *C. albicans*, *C. glabrata*, en *C. auris*, maar dat de methode ook kan worden toegepast voor het testen van andere *Candida* soorten en andere antischimmelmiddelen, zoals azolen. De belangrijkste beperking ligt momenteel in het feit dat de vereiste tijd voor de analyse langer is dan ideaal voor gebruik in een routine klinisch laboratorium. Elke nieuwe methode moet snel zijn met brede toepasbaarheid, beschikbaarheid en betrouwbaarheid, en bij voorkeur met een redelijke prijs per analyse. MBT ASTRA heeft potentieel een brede toepasbaarheid om de gevoeligheid te testen van verschillende micro-organismen tegen verschillende antibiotica en antischimmelmiddelen, en kan ook worden geautomatiseerd. Het kan hiermee de snelste AFST-methode worden voor gebruik in routine klinische laboratoria. Bovendien, de prijs van de toepassing van MBT ASTRA per monster blijft betaalbaar als de MALDI-TOF MS apparatuur beschikbaar is in het laboratorium. De bevindingen van deze studie toonden aan dat MALDI-TOF MS niet alleen een nauwkeurige methode is voor de identificatie van microben, maar dat deze ook kan worden toegepast voor AFST. Daarnaast is ook een vergelijkende studie verricht naar verschillede typeringsmethoden voor *C. auris*, en zijn mogelijkheden verkend voor het gebruik van een lactoferine peptide hLF(1-11) voor de verrijking en identificatie van *C. albicans*. Deze studie draagt bij tot een nieuwe toepassing van de MALDI-TOF MS techniek in onderzoek en gebruik in klinische laboratoria in de toekomst.

Authors' contribution to every chapter

Authors' contribution to every chapters

Chapter 1, Introduction:

M. Vatanshenassan wrote the chapter, draw and designed Figures, Tables, and finalized this chapter after revision.

T. Boekhout revised the text and approved the final version.

Chapter 2, Development of MALDI Biotyper antibiotic/antifungal susceptibility testing rapid assay (MBT ASTRA) for *Candida* species against echinocandins.

M. Vatanshenassan, K. Sparbier, M. Kostrzewa designed MBT ASTRA for *Candida* species. **M. Vatanshenassan** performed the whole experiment, optimized MBT ASTRA, analyzed data, wrote, and designed this chapter. M. Kostrzewa and T. Boekhout revised the text and approved the final version.

Chapter 3, Vatanshenassan M., Boekhout T., Lass-Flörl C., Lackner M., Schubert S., Kostrzewa M., Sparbier K. 2018. Proof of concept for MBT ASTRA, a rapid matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)-based method to detect caspofungin resistance in *Candida albicans* and *Candida glabrata*. J Clin Microbiol 56(9):e00420-18. DOI: 10.1128/JCM.00420-18.

M. Vatanshenassan, K. Sparbier, and M. Kostrzewa designed the study. **M. Vatanshenassan** optimized and setup MBT ASTRA for *Candida albicans* and *Candida glabrata* and found a new MBT ASTRA standard II for *Candida* species. **M. Vatanshenassan** analyzed data and prepared the first draft. T. Boekhout, C. Lass-Flör, M. Lackne, S. Schubert provided *Candida* strains and revised paper. K. Sparbier and M. Kostrzewa contributed to the draft revision.

Chapter 4, Vatanshenassan M., Arastehfar A., Boekhout T., Berman J., Lass-Flörl C., Sparbier K., Kostrzewa M. 2019. Rapid antifungal susceptibility testing of *Candida glabrata* isolates against anidulafungin from positive blood culture by MBT ASTRA. Antimicrob Agents Chemother 63(9):e00554-19. Doi: 10.1128/AAC.00554-19.

M. Vatanshenassan, K. Sparbier, M. Kostrzewa designed the study. **M. Vatanshenassan** performed the experiment, set up MBT ASTRA on blood samples for *C. glabrata*, analysed data, and prepared the first draft. **M. Vatanshenassan** and A. Arastehfar carried out molecular experiments. T. Boekhout, J. Berman and C. Lass-Flör provided *C. glabrata* isolates and contributed to manuscript revision. K. Sparbier and M. Kostrzewa revised the manuscript and approved the final version.

Chapter 5, Vatanshenassan M., Boekhout T., Meis JF., Berman J., Chowdhary A., Ben-Ami R., Sparbier K., Kostrzewa M. 2019. *Candida auris* identification and rapid antifungal susceptibility testing against echinocandins by MALDI-TOF MS. Front Cell Infect Microbiol 9:20. DOI: 10.3389/fcimb.2019.00020.

M. Vatanshenassan and M. Kostrzewa designed the study. **M. Vatanshenassan** performed the experiment, set up MBT ASTRA on blood samples for *C. auris*, analyzed data, and prepared the first draft. J. F. Meis, J. Berman, A. Chowdhary, and R. Ben-Ami. provided *C. auris* isolates. All authors contributed to the draft revision.

Chapter 6, Vatanshenassan M., Boekhout T., Mauder N., Robert V., Maier T., Meis JF., Berman J., Then E., Kostrzewa M., Hagen F. 2020. Evaluation of Microsatellite Typing, ITS Sequencing, AFLP Fingerprinting, MALDI-TOF MS, and Fourier-Transform Infrared Spectroscopy Analysis of *Candida auris*. J. Fungi, 146; 6(3). DOI: 10.3390/jof6030146.

M. Vatanshenassan, N. Mauder, M. Kostrzewa, and T. Boekhout designed the study. **M. Vatanshenassan** performed experiments for IR-Biotyper, MALDI-TOF MS, AFLP, data analysis, and prepared the original draft. F. Hagen and E. Then performed microsatellite assay and ITS sequencing, respectively. JF. Meis, J. Berman, F. Hagen provided *C. auris* isolates. F. Hagen, V. Robert, N. Mauder, and T. Maier contributed to data analysis. All authors, contributed to the draft revision.

Chapter 7, Vatanshenassan M., Hagen F., Boekhout T., Haas PJ., Dijksterhuis J., Brouwer C., Welling MM. 2020. Detection of *Candida* species in blood with the antimicrobial peptide hLF(1-11) using MALDI-TOF MS and fluorescent microscopy.

M. Vatanshenassan, C. Brouwer, F. Hagen, and T. Boekhout designed the study. **M. Vatanshenassan** performed the experiments, data analysis and prepared the original draft. J. Dijksterhuis provided a piece of advice to use the fluorescent microscopy and to have high-resolution figures. All authors contributed to the draft revision.

Curriculum Vitae

Personal history

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Nationality: Dutch

Languages: English, Farsi, Dutch (Intermediate)

Education

2016 - 2019 Ph.D. candidate at University of Amsterdam, The Netherlands and Bruker Daltonik GmbH, Germany.

Title of thesis: New methods for the rapid identification and antifungal susceptibility testing of clinically important *Candida* species.

2012 - 2014 Master of Science, Biomolecular Sciences, Vrije University (VU), Amsterdam, The Netherlands.

Title of thesis: DOC-1 inhibits cancer invasion in oral squamous cell carcinomas.

Title of thesis based on literature study: Antitumor immunotherapy mediated live attenuated *Salmonella* vector.

2006– 2009 Master of Science, Medical Microbiology, Tehran University of Medical Sciences, Tehran, Iran.

Title of thesis: *Trichomonas vaginalis*: investigation of a novel diagnostic method in urine samples using cysteine proteinase 4 gene and PCR technique.

2000– 2005 Bachelor of Applied Science, Medical lab technology, Tehran University of Medical Sciences, Tehran, Iran.

Work experience

Augustus 2019 - July 2020: Postdoctoral researcher - Medical mycology group at Westerdijk Fungal Biodiversity Institute - Utrecht, The Netherlands.

2016 – 2019: Early stage researcher (Ph.D. student) – Bruker Daltonik GmbH, Germany.

2015 – 2016: Research assistant - Thermo Fisher Scientific Company - Utrecht, The Netherlands.

2009 (6 months): Research assistant - Department of Medical Parasitology and Mycology, Tehran University of Medical Sciences, Tehran, Iran.

2004 – 2005: Technician - Routine clinical laboratory, Tehran, Iran.

Conferences

18 - 24 May 2019 (La Colle sur Loup, France) FEBS Advanced Lecture Course on Human Fungal Pathogens - Molecular Mechanisms of Host-Pathogen Interactions and Virulence (HFP) - Poster presentation

13 - 16 April 2019 (Amsterdam, The Netherlands) European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) - Poster presentation

30 June – 4 July 2018 (Amsterdam, The Netherlands) The International Society for Human & Animal Mycology- (ISHAM) - Oral presentation

21 - 24 April 2018 (Madrid, Spain) European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) - Poster presentation

22 - 25 April 2017 (Vienna, Austria) European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) - Poster presentation

22 - 25 April 2016 (Amsterdam, Netherlands) European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) - Poster presentation

10 - 14 April 2008 (Tehran, Iran) Sixth nationwide conference and first district congress on Parasitology and Parasitic disease.

Honors and awards

2016 Ph.D. grant awarded by the European Union (OPATHY, Project ID: 642095; Program H2020-EU.1.3.1.).

2009 Ph.D. grant awarded by Tehran University of Medical Sciences, Iran (Did not use due to emigration to The Netherlands).

2009 M.Sc. grant awarded by Tehran University of Medical Sciences, Iran.

2005 Undergraduate student prize for obtaining excellent credits by Tehran University of Medical Sciences, Iran.

Additional training

2020: GMPs, Validation, Qualification, Pharmaceutical Solutions, The Netherlands.

2019: Fundamental project management course, INVENSIVE Company, USA.

2019: Scientific writing course. Bremen University, Bremen, Germany.

2019: The way to get a patent for a novel idea and build up a company. Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands.

2018: Basic bioinformatics course. Biotechvana company, Valencia, Spain.

2018: Medical Mycology course. Hans Knöll Institut, Jena, Germany.

2017: Scientific writing course. Centre for Genomic Regulation (CRG), Barcelona, Spain.

LinkedIn https://nl.linkedin.com/pub/mansoureh-vatanshenassan/82/824/65b/nl

Research-Gate: https://www.researchgate.net/profile/Mansoureh_Vatanshenassan

List of Publications

List of publications

2020. Vatanshenassan M., Boekhout T., Mauder N., Robert V., Maier T., Meis JF., Berman J., Then E., Kostrzewa M., Hagen F. Evaluation of Microsatellite Typing, ITS Sequencing, AFLP Fingerprinting, MALDI-TOF MS, and Fourier-Transform Infrared Spectroscopy Analysis of *Candida auris*. J. Fungi 6:146.

2019. Consortium OPATHY (**Vatanshenassan M**) Gabaldón T. Recent trends in molecular diagnostics of yeast infections: from PCR to NGS. FEMS Microbiol Rev 43:517-547.

2019. Vatanshenassan M.; Arastehfar A.; Boekhout T.; Berman J.; Lass-Flör C.; Sparbier K.; Kostrzewa M. Anidulafungin susceptibility testing of *Candida glabrata* isolates from blood cultures by the MALDI biotyper antibiotic (antifungal) susceptibility test rapid assay. Antimicrob Agents Chemother 63:e00554-19.

2019. Vatanshenassan M.; Boekhout T.; Meis J. F; Berman J.; Chowdhary A.; Ben-Ami R.; Sparbier K.; Kostrzewa M. *Candida auris* identification and rapid antifungal susceptibility testing against echinocandins by MALDI-TOF MS. Frontiers in Cellular and Infection Microbiology 9:20.

2018. Vatanshenassan M., Boekhout T., Lass-Flör C., Lackne M., Schubert S., Kostrzewa M., Sparbier K. MBT ASTRA: Proof-of-concept for a rapid MALDI-TOF MS based method to detect caspofungin resistance in *Candida albicans* and *Candida glabrata*. J Clin Microbiol 56:e00420-18.

2016. Zhao L., de Hoog G.S., Cornelissen A., Lyu Q., Mou L., Liu T., Cao Y., **Vatanshenassan M**., Kang Y. Prospective evaluation of the chromogenic medium candiSelect 4 for differentiation and presumptive identification of non-*Candida albicans Candida* species. Fungal Biol 120:173-8.

2016. Bastiaens GJ., van Meer M.P., Scholzen A., Obiero J. M., **Vatanshenassan M**., *et al*. Safety, immunogenicity, and protective efficacy of intradermal immunization with aseptic, purified, cryopreserved *Plasmodium falciparum* sporozoites in volunteers under chloroquine prophylaxis: A randomized controlled trial. Am J Trop Med Hyg 94:663-73.

2010. **Vatanshenassan M**., Rezaie S., Mohebali M., Niromand N., Kazemi B., Babaei Z., Rezaeian M. *Trichomonas vaginalis*: investigation of a novel diagnostic method in urine samples using cysteine proteinase 4 gene and PCR technique. Exp Parasitol 126:187-90.

2009. Rezaeian M., **Vatanshenassan M.,** Rezaie S., et al. Prevalence of *Trichomonas vaginalis* using parasitological methods in Tehran. Iranian J Parasitol 4:43-47.

Book

2009. Daneshmandy S., **Vatanshenassan M.**, Tijanee M., Rezaeian M. Medical Parasitology. Daneshmandy publisher. First edition.

Contribution to ongoing publications

Vatanshenassan M., Hagen F., Boekhout T., Haas PJ., Dijksterhuis J., Brouwer C., Mick M. Welling MM. Detection of *Candida* species in blood with the antimicrobial peptide hLF(1-11) using MALDI-TOF MS and fluorescent microscopy (publication in preparation).

Vatanshenassan M., Boekhout T., Kostrzewa M. MALDI-TOF MS applications in mycology: from identification to an antifungal susceptibility testing (publication in preparation).

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