Malassezia Fungemia, Antifungal Susceptibility Testing and Epidemiology of Nosocomial Infections

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Core Messages

› *Malassezia* catheter-related fungemia, and invasive infections in critically ill, premature neonates and immunocompromised individuals of all ages are issues addressed in relation to risk factors, host–pathogen interactions, pathogenesis, management, and outcome. The epidemiology of drug resistance of isolates from deep tissues, biological fluids, and skin is highlighted by assessing *Malassezia* susceptibility to conventional antifungals, synthetic, and naturally occurring compounds. Yet, lack of standardization for *Malassezia* susceptibility testing restricts associations of *in vitro* with *in vivo* responses to antifungals. The chapter also describes the capacity of PCR fingerprinting, pulsed field gel electrophoresis (PFGE), and amplified fragment length polymorphism (AFLP) analysis to dependably identify nosocomial outbreaks, but concludes that robust analytical methods such as multilocus sequence typing (MLST) would be required to resolve global *Malassezia* epidemiological issues.

8.1 *Malassezia* Fungemia and Invasive Infections

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Malassezia species are rare causes of invasive infections in critically ill low-birth-weight, infants and in immunocompromised children and adults. The clinical spectrum ranges from asymptomatic infection to life-threatening sepsis and disseminated disease, with

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intravascular catheters and administration of lipid supplemented parenteral nutrition acting as the main risk factors [1–5]. Here, we review the epidemiology, risk factors, pathogenesis, clinical manifestations, diagnosis, treatment, and outcome, of invasive *Malassezia* infections in humans.

8.1.1 Epidemiology and Risk Factors

Malassezia species are well recognized as a cause of skin disorders in otherwise healthy individuals. However, information on *Malassezia* fungemia and invasive disease is limited. The overwhelming majority of invasive infections reported in the literature have been associated with *M. furfur sensu lato* and *M. pachydermatis*. *M. furfur*, which is an obligatory lipophilic yeast and a commensal in humans, has been described predominantly in conjunction with nosocomial outbreaks in neonatal intensive care units (NICU) and sporadically in severely immunocompromised patients. *M. pachydermatis*, in contrast, is a zoophilic yeast mainly associated with otitis externa and seborrhoic dermatitis in dogs. *M. pachydermatis* is only occasionally isolated from human skin, but has been implicated in nosocomial infections in hospitalized severely ill neonates [2, 3].

The first case of *Malassezia* sp. as an etiologic agent of bloodstream infection and sepsis was reported in 1981 by Redline et al. [6]; they reported a case of *Malassezia* pulmonary vasculitis in an infant receiving total parenteral nutrition via an indwelling central venous catheter (CVC). Until 1987, only few further reports of *Malassezia* fungemia in infants and adults emerged [7–11]. During the past two decades, however, there have been numerous reports on invasive *Malassezia* infections outbreaks in NICUs, particularly in neonates and infants receiving intravenous lipids [2, 12–14]. Cases have also been described in immunocompromised children and adults with CVCs, and more rarely, preceding abdominal surgery and other significant underlying conditions [15–19].

There are limited comprehensive data on the frequency of invasive *Malassezia* infections in immunocompromised patients that provide information on the overall clinical relevance of this opportunistic infection. However, studies investigating the colonization of central venous lines specifically by *Malassezia* spp. have demonstrated colonization rates of 2.4–32% in critically ill neonates [8, 20, 21] and 0.7% in unselected hospitalized adults [22]. Generally, *Malassezia* infections in bone marrow transplant (BMT) patients appear to be uncommon; as of 3,044 BMT patients, only six (0.2%) developed *Malassezia* infections, two of whom were with an involvement of the blood stream [15]. In a study of critically ill neonates, eight of 25 consecutively explanted CVCs grew *M. furfur*, and in one of the infants (4%) was found evidence of systemic infection [8]. While only routine blood cultures were utilized in the transplant patients, the study in neonates used media supplemented with olive oil, emphasizing the importance of methodological aspects in culture-based systematic epidemiological investigations.

Malassezia spp. may be isolated from the skin of 3% of healthy newborn infants, but 30–64% of hospitalized premature infants become colonized by the second week of life [5, 8, 14]. Bell et al. reported isolation of *Malassezia* yeasts identified then as *M. furfur* in 41% of critically ill newborns in the NICU, while less than 10% of hospitalized newborns

in a nonintensive care setting were colonized [23]. Likewise, Aschner et al. [8] reported that 28% of infants in an NICU were colonized in the first week of life, whereas 84% of older infants in the NICU were skin culture positive for *M. furfur*. These and other data [24, 25] indicate that colonization in neonates and infants is associated with low gestational age, admission to the NICU, and length of hospitalization [26].

Although no systematic data exist on risk factors for invasive *Malassezia* infections in immunocompromised patients beyond the neonatal age, colonization and the presence of a central line appear to be obligatory prerequisites for fungemia [1, 3, 15]. The risk factors for invasive *Malassezia* infections in adults, neonates, and infants that are summarized in Table 8.1 indicate that while invasive infections may occur sporadically, in the last decade, nosocomial outbreaks regarding neonatal *M. furfur* and *M. pachydermatis* infections have been widely reported. As shown by molecular typing methods, infants become colonized by skin contact with parents or healthcare workers, which may further transmit the organism from an infected or colonized infant to others via their hands [2, 12, 14]. In that case,

Table 8.1 Summary of predisposing factors reported in the scientific literature, and putative clinical and laboratory criteria for the diagnosis of *Malassezia* systemic infections

	Predisposing factors	Certainty of diagnosis	
		Low	High
Adults and children	Crohn's disease and various other chronic illnesses[a], CAPD, intensive care and high APACHE score, immunosuppresion, CVC, TPN with LS, long term administration of antibiotics, invasive surgical procedures	Persistent fever unresponsive to antibiotics; eucocytosis/ leucocytopenia, thrombocytopenia; identification of *Malassezia* point source nosocomial epidemic; negative microscopy and culture	Positive microscopy for yeasts with *Malassezia* morphology in Gram/ Giemsa-stained smear of buffy coat or blood drawn through the CVC or involved tissue and/or positive culture
Infants	Comorbidities of prematurity, admission to neonatal intensive care unit, use of broad-spectrum antibacterial treatment, prolonged TPN with LS, CVC, use of soybean oil-containing creams	Persistent fever unresponsive to antibiotics; leucocytosis/ leucocytopenia; thrombocytopenia; cluster of invasive *Malassezia* cases in neonatal ICU; negative microscopy and culture	Positive microscopy for yeasts with *Malassezia* morphology of buffy coat Gram/Giemsa-stained smear of blood drawn through the CVC and/or positive culture

[a]Require case controlled studies for further evaluation
CAPD chronic ambulatory peritoneal dialysis; *APACHE* acute physiology and chronic health evaluation; *CVC* central venous catheter; *TPN* total parenteral nutrition; *LS* lipid supplementation; *ICU* intensive care unit

patient-to-patient transmission may be effectively interrupted by improving hand-washing practices [12]. Other outbreak investigations have shown that *Malassezia* yeasts can also persist for prolonged time on incubator surfaces (Chap. 8.3), providing an additional source for continued transmission [27].

8.1.2 Host–Pathogen Interactions

Little is known about virulence factors and host immune responses in invasive *Malassezia* infections. *Malassezia* yeasts are able to exist in both yeast and mycelial forms; can grow under microaerophilic and anaerobic conditions; and can adhere and form biofilms on the surfaces of different materials [9, 28, 29]. They have an exceptionally thick cell wall in comparison with other yeasts with an additional lipid layer on the outside, which appears to be important for the organism's ability to suppress cytokine release, and down-regulate phagocytic uptake and killing. They also produce a range of enzymes and metabolites, including azelaic acid, which has been shown to decrease the production of reactive oxygen species (ROS) in neutrophils [28]. While these factors are in support of the general ability of the organism to cause invasive disease, their biological relevance *in vivo* remains to be elucidated.

At present, it is unclear which components of the immune system are most important in the host's defense against invasive infections. Studies examining cellular and humoral immune responses specific to *Malassezia* species in patients with superficial *Malassezia*-associated diseases and healthy controls have generally been unable to define significant differences in their immune response. *Malassezia* may stimulate the reticuloendothelial system and activate the complement cascade, but may also suppress cytokine release and down-regulate phagocytic uptake and killing. It appears that the lipid-rich external layer of the organism is pivotal in this alteration of phenotype. Thus, elucidating the nonspecific immune response to *Malassezia* species may be key to a better understanding of how these organisms live as commensals and yet can rarely cause invasive diseases [28].

8.1.3 Pathogenesis of Invasive Infections

Likely due to the sporadic nature of invasive infections, no clinical studies have addressed the immunological predisposition and responses to *Malassezia* in critically ill neonates or in immunocompromised children and adults [28]. Most patients had a serious underlying disease affecting the overlapping components of host responses; had an indwelling vascular access; and had received parenteral nutrition containing lipid emulsions (Table 8.1). As evidenced by outbreak investigations, the cutaneous commensal flora of patients or health care workers is the usual source of infection [2, 12, 14]. Apart from contamination during insertion, or following administration of a contaminated parenteral solution, catheters may become infected by migration of organism from the exit site along the outer

catheter wall or from the hub through the lumen of the catheter. Adherence of the organism to the catheter lumen with consequent biofilm production results in local replication and shedding of the organism in the blood [9, 26, 28–31]. Microbial and host factors may play a role in localizing the organisms to the catheter or in progression to fungemia and clinical sepsis [18, 20]. However, even if host defenses are able to clear the organism from the blood, the infection may not be resolved until the catheter is removed.

Similar to catheter-related candidemia [32], catheter-related *Malassezia* fungemia has been associated with administration of parenteral lipid emulsions. While the exact mechanisms of this association are unclear, it is conceivable that lipids administered through the catheter may provide a growth advantage for *Malassezia* [12, 14, 33]. On the other hand, parenterally administered lipids may negatively affect host immunity by blocking the reticuloendothelial system, reducing the generation of reactive oxygen species (ROS), which are shown to decrease phagocytosis by neutrophils *in vitro*, thus contributing to clinical disease [28].

8.1.4 Clinical Manifestations

The clinical signs and symptoms of *Malassezia* fungemia and sepsis are generally non specific. Depending on the severity of the infection, the most commonly reported symptoms in critically ill, premature infants have been fever and respiratory distress; other, less frequent symptoms include lethargy, bradycardia, hepatomegaly, splenomegaly, seizures, and cyanosis [14, 31, 34]. Respiratory distress may result from pneumonia or bronchopneumonia, with an interstitial appearance on radiography. The main laboratory findings in this setting are leukocytosis or leukopenia, and thrombocytopenia. Affected patients usually are premature, low birth weight infants with multiple comorbidities, extended hospitalization, CVCs, and parenteral nutrition including lipid emulsions [2, 7, 10, 12, 35, 36].

Catheter-associated *Malassezia* fungemia is sporadic in immunocompromised children and in adults, and consequently, clinical manifestations are not as well described as in infants. Fever appears to be universal [31]; other symptoms and findings may include chills and rigors, myalgia, nausea and vomiting, respiratory distress with or without apnea, pneumonia, leukopenia, thrombcytosis, and less frequently, leukocytosis [1, 3, 15, 30]; signs of exit site inflammation are uncommon [1]. Similar to the neonatal setting, the most common patient profile includes prolonged hospitalization, the presence of CVCs, and the use of intravenous fat emulsions [1, 3, 15, 17]. Patients reported in literature presented with a variety of underlying diseases such as bowel surgery, Crohn's disease, hemorrhagic pancreatitis, cancer and bone marrow transplantation [1, 15, 16, 18, 37, 38]. However, individual cases without the typical risk factors have been reported [39, 40].

Catheter-associated *Malassezia* fungemia may result in embolic-metastatic infection of the heart and the lungs, and less frequently, dissemination to other organs such as the skin, the kidneys, the pancreas, the liver, the spleen and the brain [26, 34, 41]. Histopathologic changes include mycotic thrombi around the tips of catheters, vegetations on the endocardium, septic inflammatory lesions in the heart and the lungs [6, 34, 42]. Reported invasive *Malassezia* infections other than fungemia include individual cases of *Malassezia* mastitis,

thrombophlebitis, sinusitis, malignant otitis externa, meningitis, septic arthritis, soft tissue abscesses, and catheter-associated peritonitis, in continuous ambulatory peritoneal dialysis patients [43–46].

8.1.5 Laboratory Diagnosis

As *Malassezia* yeasts represent an uncommon cause of fungemia and sepsis, a high index of suspicion is needed to diagnose the infection. However, while *Malassezia* fungemia has been increasingly recognized over the past two decades, its frequency may in fact be higher as the current clinical data suggest. Detection is complicated by the organism's lipid-dependent nature as most routinely used media do not support its growth [33, 43]. Use of lipid supplemented media may be warranted in certain specimens, especially if cultures appear sterile on routine media, and yeasts have been observed on microscopy. The patient group where this may be most appropriate are critically ill premature neonates receiving parenteral lipid emulsions through central venous lines. Supplementation of blood culture bottles with palmitic acid has been shown to improve recovery of *Malassezia* in this patient group [43].

Malassezia species can be detected in blood and other specimens by direct microscopic examination, by culture, and by molecular methods [33]. Examining Giemsa- or Gram-stained smears of blood or buffy coat of blood specimens (Table 8.4) obtained through the catheter is helpful and may provide the clue to culture the specimen on lipid-enriched fungal medium that supports growth of *Malazzesia* yeasts [33, 43, 47] (Chap. 2.1). Sabouraud agar supplemented with olive oil has been frequently used for this purpose. However, because only *M. furfur* and *M. pachydermatis* grow easily on this medium, other species such as *M. sympodialis* may be missed, as suspected in two cases of heart infection in Martinique with positive smear examination but negative cultures (Fig. 8.1) (N. Desbois, unpublished data). Furthermore, the time it takes to culture *Malassezia* (5 days and longer, dependent on the species) hinders rapid diagnosis, thus molecular methods can be an alternative diagnostic approach [48, 49]. A proposed PCR assay [49] used to detect blood culture-proven *M. furfur* fungemia in only four patients was of low sensitivity (25%). Therefore, improvement in sensitivity, standardization of the PCR assay, and thorough evaluation are issues that have to be resolved before employing it in the clinical setting.

8.1.6 Clinical Management

Because invasive *Malassezia* infections are rare and larger patient series are lacking, evidence-based treatment recommendations cannot be made. However, based on the association of the disease with CVCs and the ability of the organism to produce biofilms [9, 29], there is general consensus that the management of *Malassezia*-related fungemia and sepsis requires the prompt removal of any indwelling catheter, in addition to intravenous antifungal therapy, and less clear, the temporary discontinuation of parenteral nutritional lipid solutions

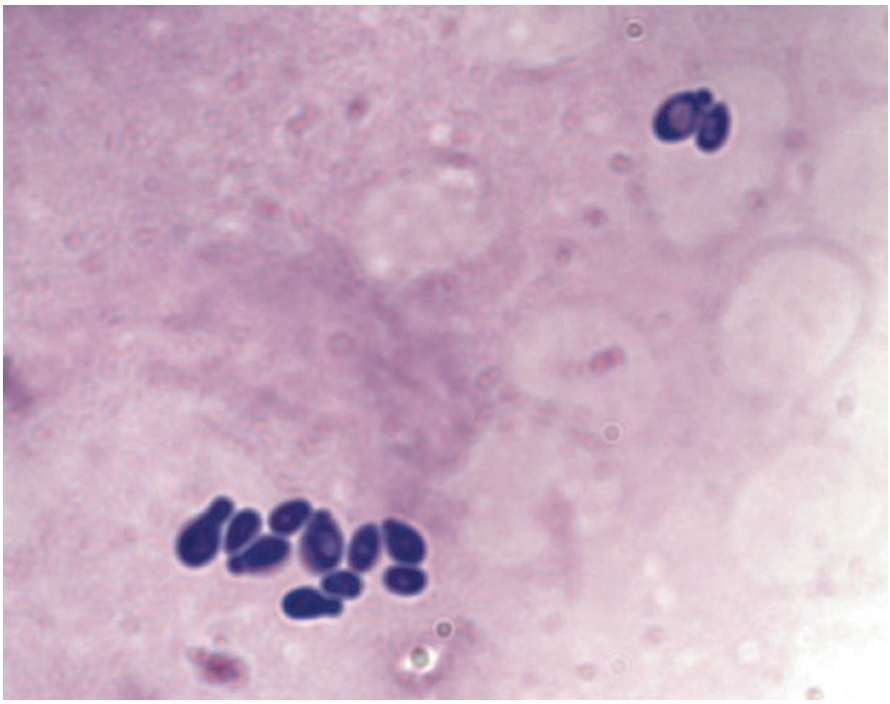

Fig. 8.1 Direct microscopy of a mitral valve apposition, showing typical ovoid *Malassezia* yeasts May-Grunwald-Giemsa stained ×1,000 (courtesy of Nicole Desbois, Fort de France, Martinique)

[33]. *In vitro* studies of superficial and invasive clinical *Malassezia* isolates consistently demonstrate susceptibility to amphotericin B and the triazoles, whereas flucytosine and the echinocandins appear to be inactive [30, 43, 50–53]. Thus, in the absence of experimental and comparative clinical data and based on the clinical experience with invasive *Candida* infections, fluconazole or voriconazole may be rational first-line options for antifungal chemotherapy with an amphotericin B product as back-up for refractory or life-threatening infections. While the duration of treatment has not been defined, we would advocate a course of 14 days of effective antifungal therapy after the last positive blood culture and catheter removal as recommended for invasive *Candida* infections [31, 32], and optional switch from initial intravenous to oral therapy depending on the individual patient's clinical response.

8.1.7 Outcome

Very little is known about the detailed morbidity and mortality of invasive *Malassezia* infections. While clearly, *Malassezia* can cause severe disease [38] and fatal cases have been reported in untreated patients [34, 54], available series of catheter-associated fungemia in premature neonates [2, 12] and in immunocompromised non-neonatal patients [1, 15] suggest low attributable mortality with appropriate management.

8.2 Antifungal Susceptibility Testing

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8.2.1 Rationale for *Malassezia* Susceptibility Testing

Antifungal susceptibility testing describes the *in vitro* response of a fungus to an antifungal agent at concentrations comparable to those achievable in blood or tissue by an antifungal drug administered at the recommended dose. Susceptibility testing is warranted for *Malassezia* yeasts, because they are involved in or trigger dermatoses and occasionally cause bloodstream and deep seated infections in humans (Chapt. 8.1). These conditions necessitate antifungal treatment. Therefore, studying *Malassezia* susceptibility to antifungal agents could identify potential resistance profiles. Overall, susceptibility data, if recorded by a standard method, could provide clinically useful information on the *Malassezia* epidemiology of resistance [55] in hospitals where ICU or NICU outbreaks have occurred, and supply critical data on the *in vitro* potency of new antifungal agents.

This section describes the current methods used to detect and quantify antifungal agent activity as well as their limitations. Furthermore, it highlights the need for standardizing susceptibility testing for *Malassezia* species. Finally, it outlines conventional and novel methods for *in vitro* investigations, bioassay testing of new antifungal agents, as well as testing new formulations of established antifungals against *Malassezia* yeasts.

8.2.2 Standard Methods and Selection of *Malassezia* Colonies for Susceptibility Testing from Mixed Blood Cultures and Skin Specimens

Standardized microdilution susceptibility testing for yeasts is a recent, yet a rapidly evolving, *modus operandi*. In addition to the current Clinical and Laboratory Standards Institute (CLSI) susceptibility testing standard, [56] the Subcommittee on Antifungal Susceptibility Testing (AFST) of the European Committee for Antimicrobial Susceptibility Testing (EUCAST) has also recently proposed a standard testing scheme for yeasts [57]. Nevertheless, these methods cannot be directly applied on *Malassezia*, as media and test conditions are still not standardized and appropriately evaluated. This is due to (a) the low rating of these yeasts as systemic pathogens, which impaired intense and methodical interlaboratory assessment of test parameters; and (b) the lipophilic nature of the majority of *Malassezia* species that has encumbered assessment of suitable growth substrates for reliable and reproducible susceptibility testing.

In addition to standardization of media and test parameters, critical in obtaining consistent susceptibility results for any tested pathogen, is selection of single species colonies grown in cultures of clinical specimens. This is of key significance in testing *Malassezia*,

as mixed species may be isolated from pathological specimens together with bacteria and *Candida* species that inhabit normal and diseased, human and animal skin. Besides mixed cultures that are obtained from skin specimens, mixed *Malassezia* sp. with *Candida* sp. or bacteria can also grow in blood cultures of high-risk (Sect. 8.1) pediatric and adult patients. In these cases, *Malassezia* colonies are frequently missed, as growth, even of *M. furfur,* may be very weak and colonies rare due to the lack of supplements required for growth of lipophilic *Malassezia* species. *Malassezia* colonies may be also missed due to the presence of antagonistic *Candida* yeasts or bacteria, the growth of which is better supported by standard blood culture media. In suspected cases of a yeast infection in high-risk patients, the choice of specialized mycological vials is critical for potential *Malassezia* co-isolation. In our experience, the BACTEC™ Myco/F lytic culture vials, with brain heart infusion broth, glycerol 0.10% w/v and Tween 80 0.0025% w/v, provide better growth support for *M. furfur* and *M. sympodialis* (A. Velegraki, unpublished observation). Therefore, differentiation of *Malassezia* cells from the prolific cells of *Candida* sp., which may co-isolate, is facilitated in the Gram-stained smears that are prepared directly from the vials. Consequently, this guides the laboratory to include modified Dixon's or Leeming-Notman agar in the subsequent subcultures to promote *Malassezia* growth.

Generally, in skin specimen cultures, it is imperative to confirm that there are single isolates of each *Malassezia* species before susceptibility testing. However, complete conventional identification (Chap. 2.1), comprising physiological and biochemical tests, as well as molecular identification are lengthy procedures (see Chap. 3.1). It would therefore be clinically realistic to pursue susceptibility testing after initial differentiation of each isolate from a mixed culture by microscopy, and to ascertain that cultures are free of bacteria by the rapid Bacto urea test as described in Chap. 2.1. Then, following subculture in *Malassezia* selective media, pure cultures of each isolate can be obtained, and susceptibility testing using single colonies can be performed in parallel with comprehensive molecular identification procedures.

8.2.3 *In vitro* Response of *Malassezia* Yeasts to Antifungal Agents

Hitherto, the susceptibility testing methodologies employed for the majority of *Malassezia* species have varied significantly, ranging from agar diffusion methods to modified CLSI broth microdilution methods using fatty acid RPMI (faRPMI) broth, modified Christensen's urea broth (mCUB) and modified Leeming-Notman (mLN) broth (Table 8.2). Yet, there are still no published trials on the *in vitro* response to established and newer antifungals of the recently recognized species *M. nana, M. caprae* and *M. equina*. Also, limited data exist on *Malassezia* species susceptibility against the expanding array of topical imidazole derivatives [58–61] and the oral triazole derivatives [50, 62, 63]. Even fewer reports cover the *in vitro* performance of broad-spectrum hydroxypyridone-derived synthetic topical antifungals [64, 65], the natural sesquiterpenoids [66], and essential oils, such as tea tree oil [67].

Despite scarcity of susceptibility data extracted thus far from nonstandardized microdilution and agar diffusion methods, there is an emerging pattern indicating possible species-dependent azole and allylamine (terbinafine) responses (Table 8.3). Even earlier [51],

Table 8.2 Antifungal drugs tested against *Malassezia* yeasts by agar dilution and broth microdilution methods and by corneofungimetry bioassays

Description of methods and media	*Malassezia* species studied	Drugs tested	References
		MIC, MFC, time-kill [66, 87] and synergy assays [88]	
Solid-phase susceptibility *in vitro* assays			
Agar dilution method in mDA, mLNA	*M. furfur, M. sympodialis, M. slooffiae, M. restricta, M. globosa, M. obtusa, M. dermatis, M. japonica, M. pachydermatis*	Amphotericin B, fluconazole, itraconazole, pramiconazole (research code: R126638), ketoconazole, luliconazole (research code: NN-502), terbinafine, rilopirox, Tea tree oil	[5, 16–18, 21, 34, 51, 59, 64, 71, 80–82, 87–90]
Liquid-phase susceptibility *in vitro* assays			
CLSI M27 A; M27 A2; M27 A3 [56] broth microdilution in faRPMI 1640, mCUB [68, 69, 91] and mLN, [52, 77] mCUB supplemented with phenol red [82] and LNB supplemented with Alamar blue [50]	*M. furfur, M. slooffiae, M. sympodialis, M. globosa, M. dermatis, M. obtusa, M. restricta, M. yamatoensis, M. japonica, M. pachydermatis*	Amphotericin B, fluconazole, itraconazole, terbinafine, voriconazole, posaconazole, albaconazole, bifonazole, miconazole, econazole, ketoconazole, luliconazole (research code: NN-502), ciclopirox olamine, xanthorrhizole, Tea tree oil	[50, 52, 61, 66, 67, 69, 70, 72, 76–80, 87, 88, 91, 99]
Bioassays			
Corneofungimetry in cyanoacrylate skin surface strippings (CSSS) [92]	*M. furfur, M. globosa, M. restricta*	2% Ketoconazole gel and ream with or without topical corticosteroid (desonide) adjunct, 1% terbinafine, pramiconazole (research code: R126638)	[84, 86, 92]

MIC minimum inhibitory concentration; *MFC* minimum fungicidal concentration; *mDA* modified Dixon's agar; *mLNA* modified Leeming-Notman; *faRPMI* 1640 fatty acid RPMI 1640; *mCUB* modified Christensen's urea broth; *LNB* Leeming-Notman broth

in vitro data generated from agar dilution methods have identified terbinafine tolerance among strains of *M. furfur, M. globosa* and *M. obtusa,* and good *M. sympodialis* terbinafine response. Similar *in vitro* species-dependent responses have been noted with the polyene, amphotericin B [63]. Specifically, the mCUB (Table 8.2) microdilution method [68, 69] identified strains of *M. globosa* and *M. restricta* with poor response to voriconazole, itraconazole and ketoconazole (MIC ≥ 8 μg/mL). The faRPMI microdilution method [63] recognized strains of *M. globosa*, *M. restricta* and *M. furfur* with poor response to fluconazole (MIC ≥ 64 μg/mL), terbinafine (MIC ≥ 1 μg/mL) and amphotericin B (MIC ≥ 8 μg/mL),

whereas increased fluconazole MIC (16 μg/mL) was noted for *M. pachydermatis* and increased terbinafine MIC (4 μg/mL) for *M. dermatis*. Interestingly, *M. globosa* strains have also been reported with MICs of itraconazole (16 μg/mL), voriconazole (≥16 μg/mL) and fluconazole (≥64 μg/mL) when tested by microdilution in mLN broth [52]. These high MIC values are indicative of tolerance to these drugs.

It is important to emphasize that the broth microdilution azole and terbinafine MIC data, compiled in Table 8.3, represent values reported by independent research groups using different test media, inocula ranging from $2.5–4 \times 10^3$ [52, 63, 70] to 10^6 [68, 69] and incubation times varying from 72–96 h [63, 70] to 168 h, [52, 68] depending on the growth rate of each *Malassezia* species. Furthermore, incubation times also depended on the ability of the test medium used in each protocol to support optimum growth for each *Malassezia* species. Likewise, lower inoculum size, in conjunction with using RPMI 1640 as test medium, could explain the lower MIC values compared to those recorded for *Malassezia* species tested in the mCUB substrate. Variable intraspecies *in vitro* responses of *Malassezia* strains to antifungals is indeed to be expected; nonetheless, definitive conclusions on potential species-specific response to antifungals can be reached once test media and conditions are evaluated using a panel of reference strains in a quality control interlaboratory exercise.

It is noteworthy that faRPMI and mCUB, used so far in broth microdilution tests, assay different properties. First, microdilution susceptibility testing in RPMI denotes growth inhibition with no color reaction designating growth. Therefore, reading MIC endpoints can be difficult due to medium turbidity caused by the lipid supplements and the button-like growth in the bottom of the microtitre plate wells [53, 63, 69]. Because of this, *M. pachydermatis* minimum fungicidal concentration (MFC) readings are following MIC readings through subcultures from each well into potato dextrose agar (PDA) plates, after incubation for 2–5 days at 32°C [69]. Application of this procedure to lipophilic *Malassezia* species, using selective media such as modified Dixon's agar may prove useful. Second, susceptibility testing using mCUB measures metabolic activity. The reported *M. pachydermatis* negative broth urease test (34.4%) among 32 veterinary isolates, [71] requires further consideration regarding the impact of mCUB in reading *M. pachydermatis* MICs. Therefore, besides screening isolates for urease activity to ensure pure *Malassezia* cultures before performing susceptibility studies, the test would also account for each pure isolate's urease activity.

Overall, the primary objective of susceptibility testing is the recognition of resistance to help selection of targeted therapy. The contribution of drug resistance in high relapse rate reported for pityriasis versicolor (PV) [62, 72, 73] is not yet evaluated. This high relapse rate is often attributed to lack of patient compliance [74] rather to treatment failure, but in the absence of a standardized susceptibility assay for *Malassezia* yeasts, correlation of *in vitro* susceptibility data with treatment outcome cannot be supported.

8.2.4 New Formulations and Newer Antifungal Agents

In the last decade, a number of new formulations of established broad-spectrum antifungals with prominent anti-*Malassezia* activity have been introduced and clinically evaluated [75]. In addition, new azoles and other compounds have so far been evaluated for either

Table 8.3 Effect of test media and inoculum size on the broth microdilution (BMD)-generated MICs for 402 [52, 63, 68–70] clinical isolates

Species/antifungal drugs	No. of strains [63]	MIC range (μg/mL)/faRPMI with low inoculum[a] [63]	No of strains [69]	MIC range (μg/mL)/mCUB with high inoculum[b] [69]	No of strains [52]	MIC range (μg/mL)/mLN with low inoculum[a] [52]	No of strains [68]	MIC range (μg/mL)/mCUB with high inoculum[b] [68]	Σ
M. furfur	12		52		74		13		151
Fluconazole		0.5–32		–		<0.125–16		–	
Itraconazole		0.03–0.06		0.03–0.5		<0.03–0.25		3.2–25	
Ketoconazole		0.03–1		0.03–1		<0.03–4		–	
Voriconazole		0.03–16		0.03–1		<0.03–0.5		–	
Posaconazole		0.03–32		–		–		–	
Terbinafine		0.03–0.5		–		–		3.2–50	
M. globosa	22		74		2		1		99
Fluconazole		0.03–0.125		–		<0.125–>64		–	
Itraconazole		0.03–0.125		0.015–≥8		0.03–16		0.8–6.3[c]	
Ketoconazole		0.03–0.125		0.0015–≥8		<0.03–05		–	
Voriconazole		0.03–0.125		0.03–≥8		<0.03–>16		–	
Posaconazole		0.03–0.06		–		–		–	
Terbinafine		0.03–0.125		–		–		0.8–6.3[c]	
M. obtusa	2		6		8		6		22
Fluconazole		2–4[d]		–		<0.125–16		–	
Itraconazole		0.03–0.25		0.03–0.125		<0.03–0.25		0.1–1.6	
Ketoconazole		0.03–0.6		0.06–0.25		<0.03–0.5		–	
Voriconazole		0.03–0.6		0.125–0.25		<0.03–1		–	
Posaconazole		0.03[d]		–		–		–	
Terbinafine		0.03–0.125		–		–		0.8–6.3	
M. restricta	2		16		–		1		19
Fluconazole		0.5–1		–		–		–	

Table 8.3 (continued)

Itraconazole		0.03[d]		0.015–>8		–		1.6–6.3[c]		
Ketoconazole		0.03[d]		0.015–≥8		–		–		
Voriconazole		0.03[d]		0.06–8		–		–		
Posaconazole		0.03[d]		–		–		–		
Terbinafine		0.125–1		–		–		6.3–25[c]		
M. sympodialis	8		50		11		7		76	
Fluconazole		0.5–16		–		<0.125–>64		–		
Itraconazole		0.03–0.06		0.015–2		<0.03–16		0.025–0.1		
Ketoconazole		0.03–0.06		0.015–4		<0.03–0.5		–		
Voriconazole		0.03–0.06		0.015–1		<0.03–>16		–		
Posaconazole		0.03–0.06		–		–		–		
Terbinafine		0.03–0.06		–		–		0.05–0.8		
M. pachydermatis	21 [63, 70]		2		–		12		35	
Fluconazole		8–16		–		–		–		
Itraconazole		≤0.03–0.25		0.03–0.125		–		0.8–6.3		
Ketoconazole		≤0.03–0.06		0.06–0.25		–		–		
Voriconazole		0.03[2]		0.125–0.25		–		–		
Posaconazole		0.125[d]		–		–		–		
Terbinafine		0.125[d]		–		–		3.2–25		
Total number of strains	67		200		95		40		402	

faRPMI fatty acid RPMI broth; *mCUB* modified Christensen's urea broth; *mLN* modified Leeming–Notman broth

[a]$2.5–4 \times 10^3$

[b]Approximately 1×10^6

[c]Range of a single isolate tested multiple times

[d]Only one value. Either one strain tested, or MICs of two strains tested identical on multiple experiments

Table 8.4 New imidazole formulations, synthetic and naturally occurring compounds evaluated for anti-*Malassezia* activity

Families of antifungal agent or compound	Antifungals	References
Topical imidazole derivatives	Anhydrous ketoconazole gel Eberconazole Lanoconazole Luliconazole	[75, 100, 93] [58] [60] [59, 61]
Oral triazole derivatives	Albaconazole Posaconazole Pramiconazole	[50] [63] [85, 97]
Topical synthetic hydroxypyridones	Ciclopirox Rilopirox	[64] [65]
Natural sesquiterpenoids	Xanthorrhizol	[66]
Essential oils	Australian tea tree oil	[67]
Cationic antimicrobial polypeptides	Cathelicidin	[83]

in vitro or *in vivo* anti-*Malassezia* activity (Table 8.4) as well as for topical preparations used in the veterinary field [76].

8.2.5 *Malassezia* Susceptibility to Synthetic and Naturally Occurring Compounds

8.2.5.1 Ciclopirox olamine

Ciclopirox is a hydrophobic broad-spectrum antifungal agent with anti-inflammatory properties that has been tested by broth microdilution against *M. furfur*. The compound is reported [77] with low MICs (range 0.001–0.25 μg/mL). Independent clinical trials have shown that ciclopirox 1% shampoo is a safe agent that improves scalp SD [78, 79]. However, there are no studies correlating the *in vivo Malassezia* yeast responses to ciclopirox shampoo with MIC values.

8.2.5.2 Rilopirox

The *in vitro* activity of rilopirox has been evaluated against *M. furfur* clinical strains from dandruff, PV, and SD, by microdilution and the agar dilution method [64]. Reported agar dilution method and microdilution MICs range from 12 to 50 μg/mL and from 16 to 128 μg/mL, respectively. The clinical relevance of the *in vitro* rilopirox performance against *Malassezia* yeasts remains to be clarified.

8.2.5.3
Xanthorrhizol

Xanthorrhizol, is an active component methanol extracted from the rhizome of *Curcuma xanthorrhiza* (Zingiberaceae) that is traditionally used in Indonesia for medicinal purposes. Its *in vitro* anti-*M. furfur* and *M. pachydermatis* activity was recently evaluated [66] by a modified CLSI microdilution method. MICs, MFCs, as well as time-kill curves have been determined. The MFCs were 5 μg/mL for *M. furfur* and 2.7 μg/mL *for M. pachydermatis,* whereas time kill readings showed that 25 μg/mL and 20 μg/mL kill 100% of the *M. furfur* and *M. pachydermatis* inoculated cells, in 5 h and in 20 min respectively. Again, the clinical relevance of these *in vitro* findings has yet to be evaluated.

8.2.5.4
Australian Tea Tree Oil

Susceptibility data on the essential oil of *Melaleuca alternifolia* are limited. Yet, there are data on the *in vitro* activity of tea tree oil, generated by agar dilution and modified CLSI microdilution methods [80] showing good *in vitro* activity against *M. furfur, M. sympodialis, M. slooffiae, M. globosa, M. obtusa* and *M. pachydermatis* [81] (MIC range for all *Malassezia* species tested by both methods: 0.03–0.25 μg/mL). It has been estimated that the recorded MICs are below the therapeutic concentrations (5–10% solutions or concentrated Tea tree oil [82]).

8.2.5.5
Cathelicidin

Indeed, the activity of cathelicidin is not tested by traditional susceptibility methods, yet, it is included herein as its quantifiable activity provides new insight into therapeutic approaches of *Malassezia*-induced skin diseases. The cathelicidin antimicrobial peptides have been isolated from many different mammalian species and play an important part in innate immune responses against bacterial infections. *M. furfur* susceptibility to synthetic and human (LL-37) antimicrobial peptides has been recently studied [83]. It has been shown that the normally expressed cathelicidins are processed to active peptides in skin biopsies of patients with PV.

8.2.6
Corneofungimetry: A Bioassay Predicting Drug Efficacy

Ketoconazole is an established, potent broad-spectrum oral antifungal with known auxiliary anti-inflamatory, antiseborrheic, and antiproliferative properties that significantly contribute in relieving the symptoms of seborrheic dermatitis (SD) (also referred to as seborrhoic eczema) (Chap. 7.1). However, its hepatotoxicity has led to the development of topical formulations (creams shampoos and powders) containing different ketoconazole concentrations. The *in vitro* activity of other topical imidazoles and oral azoles that have been developed in the recent years (Table 8.4) has been evaluated by non-standard agar dilution or microdilution

susceptibility testing (Table 8.3). Nevertheless, predicting clinical activity, even after testing susceptibility with standard methods, may be unsound due to the variable host responses to treatment and to the variable fungal load in the sites of infection. Correlating *in vitro* with *in vivo* and *ex vivo* results would therefore give a more pragmatic view on a drug's efficacy. In that respect, bioassay methods, such as corneofungimetry, can complement *in vitro* susceptibility testing to offer clinically realistic data on the topical or oral efficacy of antifungals.

Corneofungimetry [84, 85] utilizes the finding [62] that *Malassezia* yeasts can grow *ex vivo* on sebum-coated human corneocytes. By testing human stratum corneum specimens from patients undergoing topical or oral treatment, the individualized therapeutic response and the drug levels achieved in the stratum corneum can be determined.

Interestingly, corneofungimetry bioassays against *M. furfur, M. globosa*, and *M. restricta* have shown that the efficacy of the new topical 2% ketoconazole gel alone, and in combination with low-potency topical corticosteroids such as 0.05% desonide [86], which is used for the treatment of *Malassezia*-aggravated atopic dermatitis (AD also referred to as atopic eczema), instigates good clinical outcome. Similarly, good therapeutic response has been observed in patients with *Malassezia*-associated SD by a single 200 mg pramiconazole dose [84, 85]. This entailed reduction of the pre-treatment *Malassezia* yeast population at the sites of infection by approximately one-third on day seven post-treatment [85]. Assessment of the published data so far indicates that corneofungimetry bioassays can generate clinically useful data by essentially evaluating the levels achieved in the stratum corneum of patients under treatment. However, these data may not safely predict the duration of treatment for chronic or well-established infections.

8.3 Molecular Epidemiology of Nosocomial Outbreaks

Teun Boekhout

The epidemiology of nosocomial outbreaks caused by *M. furfur* and/or *M. pachydermatis* has been studied only in a few cases by molecular biology methods. An outbreak at a neonatal intensive care unit (NCIU) ward in a Dutch hospital has been studied by means of pulsed field gel electrophoresis (PFGE) and RAPD analysis [27, 94]. It is important to note that this outbreak was caused by both *M. pachydermatis* and *M. furfur,* which, as far as we know, has not been reported elsewhere. Outbreak isolates of both species were studied by RAPD using prokaryotic repeat consensus primers based on enterobacterial repetitive intergenic consensus (ERIC) or repetitive extragenic palindrome (REP) motifs and PFGE, and the resulting banding patterns were compared with a set of reference isolates from the reference collection of CBS Fungal Biodiversity Centre (www.cbs.knaw.nl). Primers ERIC IR, ERIC2, REP1R-I and REP2 generated identical banding patterns for the outbreak isolates, when compared to those of the reference strains that showed some variation [27]. Karyotyping of intact chromosomes with PFGE, however, identified minor chromosomal length variation in outbreak and reference isolates alike, suggesting involvement of different strains in the outbreak. This supposition, however, was challenged by the RAPD-generated data that showed no genetic diversity in the outbreak isolates, whereas some diversity was present in the reference strains.

It was therefore concluded that the outbreak was, most likely, caused by a single strain. As also discussed in Chap. 3.1., it seems that PCR typing is a more sensitive epidemiological tool than PFGE. The outbreak was controlled by improving hygiene measures in the NICU. In this case, no attempt was made to identify the source of the outbreak.

In another *M. pachydermatis* outbreak in a NICU in the USA, pulsotyping of HaeIII digested chromosomes indicated that the banding patterns of all 15 isolates obtained from the neonates were identical, and these also matched those from isolates obtained from a health care worker and those from his pet dog. The authors concluded, although with some uncertainty, that *M. pachydermatis* was most likely introduced into the ward through the colonized hands of dog-owner health care workers who subsequently transmitted it from patient to patient. Similar to the outbreak in the Dutch hospital, improvement of hygiene measures, such as meticulous hand washing practices and use of a new pair of disposable gloves while caring for each neonate, eliminated the outbreak [12]. Contrary to the evidence for the aforementioned outbreaks that have been attributed to a single strain, two other outbreak reports that occurred in two distinct geographic locations indicate involvement of multiple *M. pachydermatis* strains.

RAPD typing of 14 catheter-related outbreak isolates from different patients in a Sweedish NICU, demonstrated genetic heterogeneity, whereas *M. pachydermatis* isolates from different body sites of the same neonate yielded the same banding pattern. The investigators also assumed that the neonates were either initially colonized by *M. pachydermatis* from the skin of their parents, or from the hands of nursing staff, and that, subsequently, infection occurred via permanent catheters [2]. Using amplified fragment length polymorphism (AFLP) analysis (Fig. 8.2) of nine isolates of *M. pachydermatis,* originating from a

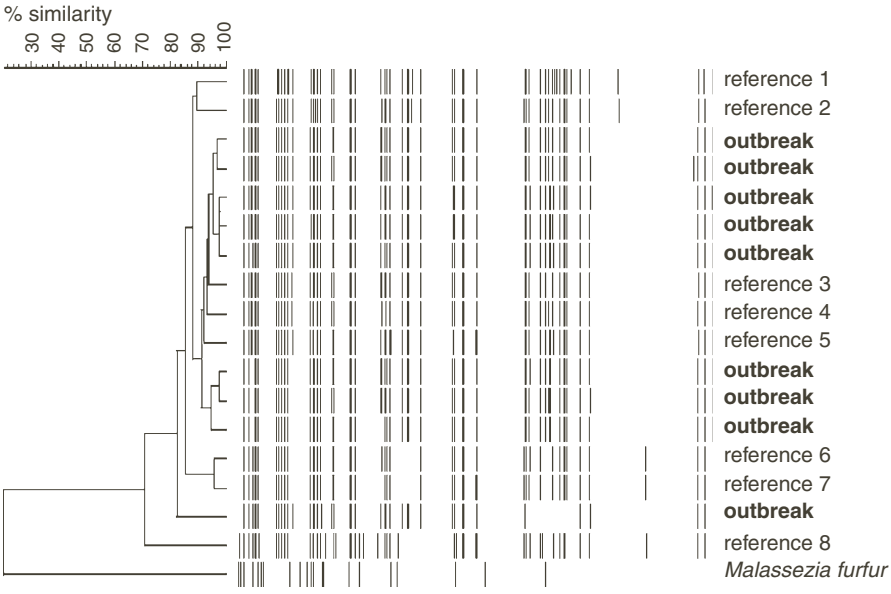

Fig. 8.2 Genetic variation, as determined with amplified fragment length polymorphism (AFLP), of a series of isolates from a nosocomial outbreak and reference strains from a culture collection. Note that the amount of genetic variation is similar to that of the genetically nonrelated reference strains, thus indicating that multiple isolates were involved in the outbreak

nosocomial outbreak in a USA-based hospital, some genetic diversity was observed, similar to the level present in a number of reference isolates obtained from dogs, but from different geographical regions [95].

8.4 Conclusions

Over the past two decades, *Malassezia* has been increasingly recognized as cause of catheter-associated fungemia in critically ill premature neonates and immunocompromised individuals of all ages. However, since many routine blood culture systems do not effectively support its growth *in vitro*, the incidence will most likely be even higher as current clinical data suggest. A high index of suspicion is required to recognize *Malassezia* species as cause of invasive infections. Microbiological diagnosis still relies on the growth of the organism in appropriate culture media, but molecular methods offer some hope for improved detection, thus contributing in collecting consistent epidemiological data in the near future. While no evidence-based treatment recommendations can be made, systemic therapy with antifungal triazoles or amphotericin B and removal of indwelling devices are considered the cornerstones of clinical management.

As *Malassezia*-induced or exacerbated infections require systemic and/or topical antifungal chemotherapy, susceptibility testing represents a *sine qua non* clinical laboratory procedure. Thus far, testing protocols by agar dilution and broth microdilution formats imply possible *M. restricta, M. globosa* and *M. sympodialis* resistance to certain azole drugs. Systematic studies correlating clinical outcome with susceptibility results would elucidate this point, as the existing sporadic data do not sponsor definitive conclusions. However, standardization is needed, for it is obvious that appropriate validation is required before adopting a susceptibility assay intended for patient care and welfare. Susceptibility testing is a highly controlled procedure. As with any assay, its stochastic nature subjects it to random strain and procedures-related events, which may lead to erroneous results. Clinical relevance of susceptibility results for topical and oral agents targeting the skin can be assessed with standardized susceptibility testing procedures, co-evaluated with pharmacodynamic and pharmacokinetic studies in natural systems such as the human stratum corneum. Besides the impact of *Malassezia* susceptibility testing assays in the clinical domain, routine performance of standardized testing would also provide useful information on the epidemiology of resistance to antifungal agents in the hospital and in the community.

Evidence thus far supports the hypothesis that *Malassezia*-related nosocomial outbreaks in neonatal wards can be caused by either a single or multiple *M. pachydermatis* strains. The only *M. furfur* outbreak that was studied by molecular means was, most likely, caused by a single strain.

From the foregoing discussion, it is clear that different methods have different resolution capacities, and, consequently, the generated epidemiological data are interpreted accordingly. Based on our experiences, PCR-fingerprinting methods using ERIC or REP primers, as well as AFLP may be preferred over PFGE. The main disadvantage of these

fingerprint methods is the lack of time-spatial reproducibility thus impeding objective comparison of the results. A promising method, which, unfortunately, has not yet been used in epidemiological studies of *Malassezia*-related outbreaks, is the multilocus sequence typing (MLST) in which several housekeeping genes (usually up to 7–10) are being sequenced across all available isolates [96–101]. We foresee the future development of a MLST-typing scheme of *Malassezia* species that may be coordinated by the ISHAM working group on *Malassezia*. The main advantage of MLST is that the results are reliable and reproducible. Another advantage is that storing of the MLST data in a central depository, such as the http://www.mlst.net, will make it possible for other investigators, epidemiologists, and clinicians to compare isolates from their studies. Finally, if a sufficient body of epidemiological data is generated, our global *Malassezia* epidemiological perspective will improve.

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