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Blood culture procedures and diagnosis of *Malassezia furfur* bloodstream infections: Strength and weakness

Roberta Iatta[1], Michela Battista[2], Giuseppe Miragliotta[3], Teun Boekhout[4,5], Domenico Otranto[1] and Claudia Cafarchia[1,*]

[1]Dipartimento di Medicina Veterinaria, Università degli Studi di Bari "Aldo Moro", Italy, [2]U.O.C. Microbiologia e Virologia, Policlinico, Bari, [3]Dipartimento Interdisciplinare di Medicina, Università degli Studi di Bari "Aldo Moro", Italy, [4]Westerdijk Fungal Biodiversity Centre, Uppsalalaan, Utrecht, The Netherlands and [5]Institute of Biodiversity and Ecosystem Dynamics (IBED), University of Amsterdam, Amsterdam, The Netherlands

*To whom correspondence should be addressed. Claudia Cafarchia, DBSci, Dipartimento di Medicina Veterinaria, Università degli Studi di Bari "Aldo Moro", Str. Prov. per Casamassima km 3, 70010 Valenzano, Bari, Italy.
Tel: +39 080 4679834; Fax: +39 080 4679839; E-mail: claudia.cafarchia@uniba.it

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Abstract

The occurrence of *Malassezia* spp. bloodstream infections (BSIs) in neonatal intensive care unit was evaluated by using pediatric Isolator, BacT/Alert systems and central venous catheter (CVC) culture. The efficacy of BacT/Alert system in detecting *Malassezia* was assessed by conventional procedures, culturing 1 ml of bottle content before incubation and by studying the survival of *Malassezia* spp. strains in BacT/Alert bottles. Of the 492 neonates enrolled, blood was collected by pediatric Isolator (290 patients; group I) or by BacT/Alert bottles (202 patients; group II). The survival of *Malassezia furfur* and *Malassezia pachydermatis* in BacT/Alert bottles was evaluated by culturing the inoculum suspension (from 10^6 to 10 colony-forming units, cfu/ml) and assessing the cfu/ml for 15 days. In total, 15 *Malassezia* BSIs were detected, of which six (2.1%) from both blood and CVC culture in Dixon agar (DixA) in patients belong to group I (blood collected by paediatric Isolator tube) and nine (4.4%) only from CVC culture in DixA in patients of group II (blood collected by BacT/Alert bottle). Only one patient (0.5%) from group II scored positive for *M. furfur* also by culturing in DixA 1 ml blood content of BacT/Alert bottle before incubation in BacT/Alert system. *M. furfur* population size in BacT/Alert bottles decreased during the incubation time, whereas that of *M. pachydermatis* increased. The BacT/Alert system detected *M. pachydermatis* even at very low concentration (i.e., 10 cfu/ml) but not any positive blood culture for *M. furfur*. For a correct diagnosis of *Malassezia furfur* BSI, the blood should be culture in lipid-enriched fungal medium, and the BacT/Alert

system implemented by adding lipid substrates to increase the method sensibility. Finally, CVC cultures on lipid-supplemented media may be proposed as a routine procedure to diagnose the *Malassezia* fungemia.

Key words: *Malassezia furfur*, bloodstream infections, diagnosis, BacT/Alert system.

Introduction

Malassezia fungemia by *Malassezia pachydermatis*, *Malassezia furfur* and *Malassezia sympodialis* [1–6] may be associated with nosocomial outbreaks in immunocompromised patients.[7] Though occasionally reported, this infection has attracted the attention of scientists for the inherent difficulties in its diagnosis and treatment.[8–14] A diagnostic survey on bloodstream infections (BSIs) resulted in a higher prevalence of *M. furfur* (2.1%) than *Candida* spp. (1.4%) suggesting that *Malassezia* BSIs might be underestimated, due to improper diagnosis.[1] BSIs caused by lipid-dependent *Malassezia* species are detected either using systems pre-enriched with lipid substrates[15,16] or by direct isolation using the Isolator system (DuPont Co., Wilmington, DE, USA).[1,17,18] Although the automated blood culture system BacT/Alert (bioMérieux, Marcy l'Etoile, France) is worldwide used in many laboratories for the detection of bacterial and fungal pathogens, its failure to detect *M. furfur* has been reported through a retrospective clinical review.[17] In this study, the occurrence of *Malassezia* spp. BSIs in a neonatal intensive care unit (NICU) has been evaluated by using two different blood culture procedures (i.e., pediatric Isolator and BacT/Alert systems) and by culturing blood and central venous catheter (CVC) tips onto specific medium (i.e., Dixon agar). The survival of *Malassezia* spp. strains in BacT/Alert bottles was also evaluated, and the efficacy of BacT/Alert system in detecting positive samples was correlated with the *Malassezia* spp. population size.

Methods

Human patients and *Malassezia* spp. BSIs diagnosis

Blood and CVC were collected from 492 neonates, of which 290 (group I) enrolled during a 1-year survey on yeasts fungemia[1] conducted from July 2011 to July 2012 and 202 (group II) enrolled from January 2016 to January 2017. All patients displayed at least one of the following clinical signs: respiratory distress, elevated or depressed leukocyte count, increased C-reactive protein levels (CRP $\geq$5 mg/l), abdominal distension, or thrombocytopenia. Blood samples were collected by lysis centrifugation tubes (pediatric Isolator 1.5-ml system, DuPont Co., Wilmington, DE, USA) from 290 patients of group I and by BacT/Alert pediatric (PF) bottles from patients of group II. For *Malassezia* spp. BSIs diagnosis, pediatric Isolator tubes were processed according to the manufacturer's recommendations by culturing 0.35 ml of Isolator tube content onto Dixon agar containing 0.5% chloramphenicol (DixA). BacT/Alert PF bottles were processed according to the manufacturer's recommendations with some modifications. In particular, before bottle incubation (i.e., T_0), 1 ml of bottle content (i.e., sampled blood + culture medium) was aseptically collected, centrifuged, and the pellet inoculated onto DixA, then incubated at 32°C for 3 days. Bottles were incubated at 35°C into the BacT/Alert system and monitored for up to 15 days for fungal growth. When a positive culture was indicated by the system, 0.2 ml of BacT/Alert bottle content was subcultured onto DixA.

CVCs from both groups I and II were cultured by rolling the CVC tip[19] onto DixA immediately after their sampling and the inoculated fungal medium was incubated at 32°C for 15 days and daily examined, as routine diagnostic method.[20] Following preliminary yeast identification using an automated system (Vitek2; bioMérieux, Craponne, France), *Malassezia* spp. isolates were characterized based on morphological and biochemical features (e.g., catalase reaction, ability to assimilate Tweens (i.e., 20, 40, 60, 80), growth on PEG-35 castor oil, and β-glucosidase activity).[20] *Malassezia* spp. isolates were identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) as previously reported.[21] Because of the observational nature of this study a written informed consent from the parents or guardian of patients was obtained according to the current Italian legislation (Art. 81-D.Lgs.vo n.196/2003).

Efficacy of the BacT/Alert system in detecting *Malassezia* BSI

The efficacy of the BacT/Alert system in detecting *Malassezia* BSI was assessed testing 15 *Malassezia* strains (i.e., n. 7 from blood and n. 8 from CVC) from 15 patients, and one *M. pachydermatis* (ITS-1 GenBank Accession number DQ915503) from the skin of a dog with dermatitis, used as control being the only non–lipid-dependent species within the genus.[22] For each strain, an inoculum suspension (IS), corresponding to 1–5 $\times$ 10^5 colony-forming units (cfu/ml) in 5 ml of sterile distilled water, was prepared using 5-day-old colonies and 1 ml transferred into a 20-ml culture medium BacT/Alert PF bottle containing 3 ml of

blood obtained from healthy volunteers. Two bottles for each test were processed either by automated BacT/Alert system or by quantitative plate count of cfu/ml on DixA. For the control cultures, 1 ml of the IS was transferred into 23 ml of Dixon broth, incubated at 35°C in a shaking incubator and monitored for the *Malassezia* population size under the same conditions. The bottles were incubated in the BacT/Alert automated system at 35°C for 15 days, and cfu/ml was evaluated before incubation (i.e., T_0), after 1 day and every 2 days up to 15 days of incubation (i.e., from T_1 to T_{15}).

An IS corresponding to 1–5 × 10^8 cfu/ml and five consecutives 1:10 dilutions (i.e., D_1-D_5) in sterile distillated water for both *M. furfur* (GenBank Accession n. KF682274), and *M. pachydermatis* (GenBank Accession number DQ915503) strains were performed to assess the sensitivity of the BacT/Alert system in detecting *Malassezia* BSI in relationship to *Malassezia* population size. In particular, 1 ml of the IS and of each dilution was transferred into a 20-ml culture medium BacT/Alert PF bottle containing 3 ml of blood and processed as above. The experiment was also performed using BacT/Alert FA bottles (i.e., those employed for adult patients) under the same conditions.

Statistical analysis

All the experiments were performed in duplicate and repeated twice on different days. The results were plotted and reported as mean values of Log_{10} cfu/ml. The population size of *Malassezia* spp. in the control medium (i.e., Dixon broth) and in the BacT/Alert bottles, at each time point, were compared by using the *t* test for paired samples. Statistical significance was set at $P \leq 0.05$.

Table 1. Number and percentage of infants from Group I (blood collected by lysis centrifugation tube) and Group II (blood collected by BacT/Alert pediatric - PF bottles) scored positive for *Malassezia* bloodstream infections by using blood and central venous catheter (CVC) cultures.

	Positive /Total (%)		
Diagnosis	Group I	Group II	Total
Blood in BacT/Alert system	—	0/202 (0)	0/202 (0)
Blood in Dixon agar at T_0	6/290 (2.1)	1/202 (0.5)	7/492 (1.6)
CVC in Dixon agar	6/290 (2.1)	9/202 (4.4)	15/492 (3.1)

Results

Of the 15 *Malassezia* BSI-positive samples (3.1%), *M. furfur* was isolated from blood and CVC cultures in six patients (2.1%) from group I (blood in pediatric Isolator tube) and from CVC cultures in nine patients (4.4%) from group II (blood in BacT/Alert bottle), of which one was also positive, at T_0 for 1 ml blood culture from the BacT/Alert bottle (Table 1). The BacT/Alert instrument gave no positive

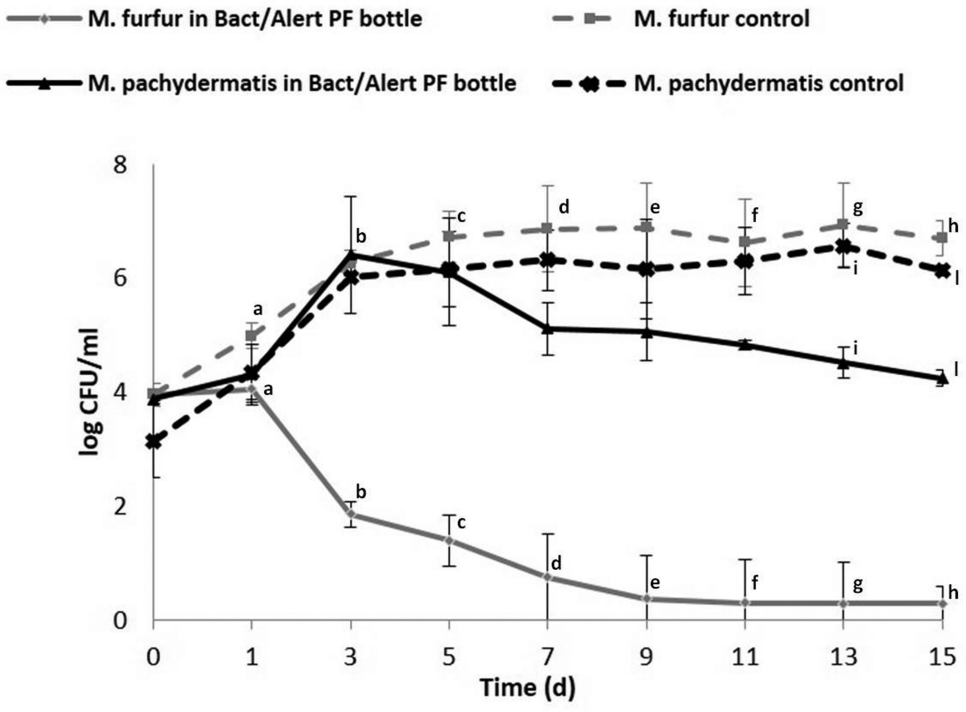

Figure 1. Population size of *Malassezia furfur* and *Malassezia pachydermatis* expressed as Log_{10} of Colony Forming Units - CFU/ml in BacT/Alert PF bottles and in Dixon broth as control after different days of incubation. The statistically significant differences are indicated with the same letters.

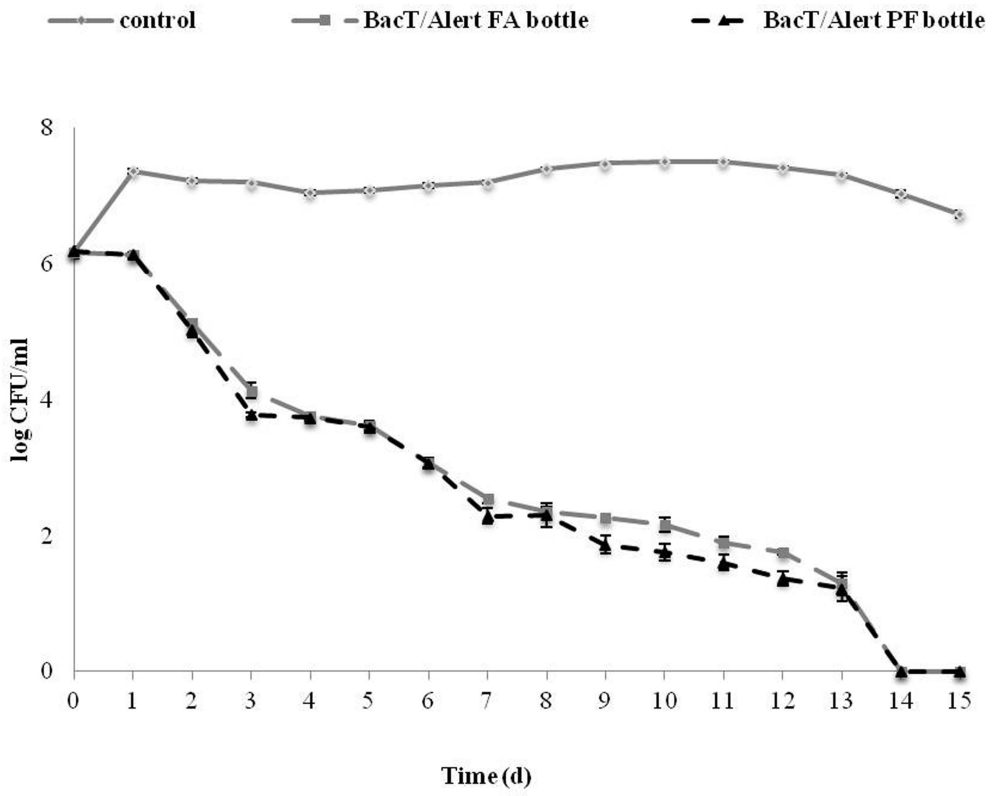

Figure 2. *Malassezia furfur* growth at different time points into BacT/Alert FA and PF bottles and in Dixon broth as control.

signal. All *Malassezia* strains were identified by biochemical features and MALDI-TOF MS as *M. furfur* (data not shown). The *M. furfur* strains isolated from blood and CVC culture in six patients from group I showed the same MALDI-TOF MS profile.[21]

In control cultures, *M. furfur* and *M. pachydermatis* population size increased during the incubation from 2–9 × 10^3 cfu/ml (Log_{10} cfu/ml = 3.15–3.96) at T_0 to the stationary phase (Log_{10} cfu/ml = 6.01–6.26) at T_3 (Fig. 1). In BacT/Alert PF bottles, *M. furfur* population size decreased from 9 × 10^3 cfu/ml (i.e., Log_{10} cfu/ml = 3.96) at T_0 to zero cfu/ml at T_9 with values significantly lower in BacT/Alert bottles than that in control cultures after 1 day of incubation ($P < 0.05$) later on (Fig. 1). Conversely, at the same conditions, *M. pachydermatis* population size increased from 7.6 × 10^3 cfu/ml (Log_{10} cfu/ml = 3.88) at T_0 to 2.5 × 10^6 cfu/ml (Log_{10} cfu/ml = 6.41) at T_3 (Fig. 1). In the BacT/Alert bottles the population size of *M. pachydermatis* was significantly lower than that in control culture after T_{13} (i.e., Log_{10} cfu/ml = 4.51 in BacT/Alert bottles *vs* Log_{10} cfu/ml = 6.57 in Dixon broth) (Fig. 1). In the event of a population size of 7.6 × 10^3 cfu/ml (Log_{10} cfu/ml = 3.88), *M. pachydermatis* positive samples were revealed by the BacT/Alert system after 30 hours of incubation.

M. furfur inoculum dilutions (i.e., from 10^6 to 10 cfu/ml) decreased during the incubation from 1.2 × 10^6 cfu/ml (Log_{10} cfu/ml = 6.12) at T_0 to zero cfu/ml within 14 days (T_{14}) (Fig. 2) and from ~10 cfu/ml at T_0 to zero cfu/ml after 3 days (data not shown), in both BacT/Alert FA and PF bottles. Conversely, all *M. pachydermatis* inoculum dilutions increased during the incubation reaching the stationary phase in both BacT/Alert FA and PF bottles and the BacT/Alert system revealed *M. pachydermatis* positive samples after 2 days of incubation regardless of the population size at T_0.

Discussion

The results of this study indicate that *Malassezia* fungemia in preterm infants might occur with a prevalence ranging from 2.1% to 4.4%, according to the diagnostic procedures employed, therefore suggesting the relevance of a proper diagnosis. The Isolator system gave better results in detecting *M. furfur* fungemia than automated system in the case of lipid-supplemented medium (i.e., Dixon agar) used for culturing this yeast.[17]

However, even if the beneficial effect of the Isolator system for the detection of *M. furfur* or dimorphic fungi (i.e., *Histoplasma* spp.) is documented,[23] the reduced use

of this system for fungemia diagnosis, has allowed a reduction in costs, sample processing times and false-positive.[17] In this study, we evaluated the efficacy of the BacT/Alert system in detecting *M. furfur* and *M. pachydermatis* BSIs, through a direct culturing procedure of bottle content at T_0 and by incubation of BacT/Alert bottles in the automated system. However, though blood was immediately cultured on specific medium (i.e., DixA), *M. furfur* was detected only in one case probably as an effect of (i) the low *Malassezia* population size in blood (usually lower than 10^2 cfu/ml)[24] and of (ii) yeast dilution in the automated BacT/Alert bottles containing at least 20 ml of tryptic soy broth as medium. Conversely, the finding of one blood sample positive for *M. furfur* might be due to the presence of detectable yeast population size in the blood of the hospitalized patient. Therefore, the absence of lipid supplementation in the medium of BacT/Alert bottles is most likely the cause of *Malassezia* growth failure. This was also confirmed by the fact that *M. pachydermatis* grows in the BacT/Alert bottles being easily detected by the system.

The *Malassezia* population size does not affect the sensitivity of the system since at T_0 *M. pachydermatis* was detected at low density (i.e., 10 cfu/ml) but not *M. furfur*, even at high density (i.e., 10^6 cfu/ml). Therefore, since the instrument gave a positive signal after 2 days, regardless the initial load of *M. pachydermatis*, the increase of one logarithm cfu/ml could be enough for a positive diagnosis. Finally, the highest prevalence of *Malassezia* BSI detected using CVC culture in DixA suggests that this procedure should be used to diagnose *Malassezia* CVC related fungemia when other causes of sepsis have been ruled out.[25,26]

Although new methods might be used for the diagnosis of *Malassezia* BSI,[27] the blood culture BacT/Alert system remains one of the most common system used in many laboratories for the detection of bacterial and fungal pathogens. Therefore, for a correct diagnosis of *M. furfur* BSI the blood culture in lipid-enriched fungal medium, such as Dixon agar, should be used for the diagnosis and the BacT/Alert system implemented to increase the sensibility in the diagnosis of *M. furfur* BSIs. Finally, the prevalence of 4.4% of *Malassezia* infection registered in patients of group II and gained by CVC cultures on lipid-supplemented media might suggest that this approach may be proposed as a routine procedure to diagnose the infection. A proper diagnosis will further contribute to scientific knowledge about the clinical significance of *M. furfur* and its implication in the management of immunocompromised hosts.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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