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Methods for Optimal Recovery of *Malassezia furfur* from Blood Culture

MARIO J. MARCON,[1,2]* DWIGHT A. POWELL,[3] AND DIANE E. DURRELL[3]

Departments of Pediatrics[3] and Pathology,[1] Ohio State University, and Department of Laboratory Medicine, Children's Hospital,[2] Columbus, Ohio 43205*

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***Malassezia furfur*, a recently described agent of catheter-associated sepsis, requires long-chained fatty acids for in vitro growth. To determine optimum conditions for recovery of the organism from blood culture, we compared a radiometric (BACTEC) with a lysis centrifugation-direct agar plating (Isolator) blood culture method. When blood culture isolates of *M. furfur* were suspended in phosphate-buffered saline and used as inocula, the BACTEC system detected the organisms radiometrically only when bottles were supplemented with lipid compounds; detection was often delayed (≥72 h) when small inoculum sizes were tested. The Isolator pediatric tube system detected growth of *M. furfur* within 48 h of plating onto a lipid-supplemented agar medium. Simulated blood culture experiments showed that the addition of whole human blood and Intralipid to the BACTEC 6B bottle was associated with rapid lysis of erythrocytes, accumulation of a chocolate brown sediment in the bottles, and fungicidal activity to the *M. furfur* isolates. In contrast, suspensions of *M. furfur* in whole human blood were stable for at least 8 h in Isolator tubes and quantitatively recoverable after plating onto agar. Of the two systems studied, the Isolator tube system provided a more suitable procedure for isolation of *M. furfur* from blood culture.**

Recently published data from several pediatric institutions describe the association of *Malassezia furfur*, a lipophilic, normal skin flora yeast, with deep-line catheter-related systemic infection in neonates and infants receiving parenteral fat emulsion therapy (7, 7a, 10, 12–14). In these reports, *M. furfur* was isolated from blood collected via deep-line catheters or cultured directly from the catheter at the time of removal; in only one study was the organism isolated from venipuncture blood cultures (14).

In our laboratory, *M. furfur* was first isolated from a catheter blood culture after observing budding yeast cells on "blind" Gram-stained smears of blood culture broth followed by subculture onto blood agar plates (10). The blood bottles did not show overt turbidity or other visible signs suggesting microbial growth. The yeast produced only very tiny colonies on agar unless a few drops of olive oil were placed onto the agar surface. Subsequently, additional isolates of *M. furfur* were recovered from catheter blood cultures by: (i) radiometric detection in BACTEC (Johnston Laboratories, Inc., Towson, Md.) 6B bottles because of a low positive growth index reading (≥30) or an increase of ≥10 growth index on consecutive daily readings; (ii) blind Gram stain smear of blood culture bottles; or (iii) blind subculture onto media with olive oil dropped onto the agar surface.

Because of its growth dependence on exogenous long-chained fatty acids, *M. furfur* grows slowly or not at all in routine microbiologic broth or agar media without lipid supplementation (8, 9). For this reason, recovery of *M. furfur* from routine blood cultures is unlikely unless the patient's blood contains sufficient lipid to initiate in vitro growth in blood culture bottles and subculture into a lipid-containing medium is performed. This raises the concern that *M. furfur* sepsis may be undetected.

To assess the incidence of *M. furfur* catheter-related sepsis, a reliable and widely applicable blood culture system must be utilized. The system should be able to isolate small numbers of organisms in a rapid fashion and should not add significantly to the cost and time required to perform the culture. We chose to study the BACTEC system with lipid supplementation of 6B aerobic broth media and compare it to direct agar plating of blood using the Isolator (Du Pont Co., Wilmington, Del.) pediatric blood culture system, which has been reported to yield excellent isolation of yeasts from blood culture (2, 3).

MATERIALS AND METHODS

Source of reagents. The following reagents and chemicals were obtained from the manufacturers indicated: Tween 80, Sigma Chemical Co., St. Louis, Mo.; glycerol monosterate, Polysciences, Ltd., Warrington, Pa.; Triton X-100, Fisher Scientific Co., Pittsburgh, Pa.; oleic acid, Baker and Adamson, Morristown N.J.; Liposyn, Abbott Laboratories, Chicago, Ill.; and Intralipid, Cutter Laboratories, Berkeley, Calif. Liposyn and Intralipid were purchased as 20% fat emulsions containing the following compounds per 100 ml: vegetable oil mixture, 20 g; egg phosphatide, 1.2 g; glycerol, 2.5 g; and distilled water to volume. The vegetable oils in Liposyn and Intralipid are derived from safflower and soybean, respectively, and differ in their fatty acid composition. In all experiments, concentration of these products is expressed as final percentage (by volume) of fat emulsion.

Source, storage, and growth of *M. furfur* on agar media. A total of eight clinical isolates of *M. furfur* were used during this study. All were obtained from blood drawn via deep-line catheters or isolated from catheter tip segments at the time of removal from patients receiving fat emulsion therapy. The clinical presentation of five of these patients has been described (10). Isolates were identified as *M. furfur* by their growth requirement for olive oil on Sabouraud dextrose agar, colonial morphology, and microscopic appearance of collarettes on unipolar budding yeast cells (8). They were stored frozen at −70°C in sheep blood. Growth on solid agar medium was accomplished by subculture onto Sabouraud

* Corresponding author.

dextrose agar medium followed by the addition of 2 to 4 drops of sterile olive oil and incubation at 35°C. Alternatively, the organism was grown on a supplemented glucose-yeast extract-peptone agar (GYP-S) medium with 2% olive oil, 0.25% glycerol monosterate, and 0.1% Tween 80 incorporated into the agar (5). Quantitative colony count is more easily accomplished on GYP-S medium due to the lack of significant amounts of olive oil on the agar surface (4).

Preparation of broth suspensions of *M. furfur*. Broth suspensions of *M. furfur* were prepared by inoculating surface growth from 3-day-old GYP-S agar plates into tubes containing phosphate-buffered saline (PBS; 0.01 M, pH 7.4) supplemented with 0.1% Triton X-100 (PBS-Triton; to eliminate aggregation of the organisms). Suspensions of approximate CFU per milliliter were prepared by standardization against McFarland turbidimetric standards or by spectrophotometry at 540 nm. In all experiments, density of yeast cells was confirmed by quantitative plating onto GYP-S agar.

Growth experiments in BACTEC 6B bottles. BACTEC 6B bottles were supplemented with various lipid compounds, inoculated with 1-ml suspensions of *M. furfur* in PBS-Triton, and incubated at 35°C with shaking. Each bottle was sampled once every 24 h for 7 days on the BACTEC 460 instrument to measure radiometric growth index values. In certain experiments, 0.1-ml samples were removed from the bottles and quantitatively plated onto GYP-S agar to determine colony count.

Stability and recovery of *M. furfur* in Isolator tubes. Human blood from adult volunteers was freshly drawn by venipuncture, seeded with *M. furfur*, and immediately dispensed in 1.5-ml volumes into Isolator pediatric tubes. Control tubes were seeded with similar suspensions of *M. furfur* in PBS-Triton. At various times during incubation at room temperature, 0.1-ml samples were repeatedly withdrawn from the tubes, plated onto GYP-S medium, and incubated at 35°C for 3 days to determine colony count.

Simulated blood culture experiments. Blood was freshly drawn by venipuncture from human volunteers and rapidly added to previously prepared suspensions of *M. furfur* in PBS-Triton (blood/suspension ratio, 99:1). The resulting mixture was immediately vortexed, and 1- or 1.5-ml volumes were inoculated into BACTEC 6B bottles previously supplemented with a lipid source or Isolator pediatric tubes (no lipid supplementation), respectively. BACTEC bottles were incubated at 35°C with shaking for a total of 7 days. At 24-h intervals, radiometric growth index values were measured, and 0.1-ml samples of broth were removed and quantitatively plated onto GYP-S agar to determine colony count. Pediatric Isolator tube blood was withdrawn within 1 h of inoculation, and 0.5-ml aliquots were plated in duplicate onto GYP-S agar either directly or following serial 10-fold dilution. The plates were incubated at 35°C and examined daily.

RESULTS

In preliminary experiments, we showed that unsupplemented BACTEC 6B bottles did not support the ^{14}C metabolism of *M. furfur* following inoculation of 10^3 CFU (11). However, *M. furfur* did yield positive growth index values (≥30) within 72 h after inoculation into BACTEC 6B bottles supplemented with a variety of sterile vegetable oils (2%, vol/vol) or oleic acid, Tween 80, Liposyn, or Intralipid (1%, vol/vol). Maximum growth index values were obtained in bottles supplemented with the latter three lipid sources, and they were therefore selected to further assess their ability to support ^{14}C metabolism and growth in BACTEC bottles.

TABLE 1. Effect of lipid concentration on metabolism of ^{14}C-labeled substrates by *M. furfur* in supplemented BACTEC 6B broth[a]

Labeling period (h)	Growth index in lipid-supplemented bottles[b]									
	None	Tween 80			Intralipid			Liposyn		
		0.25%	1%	8%	0.05%	0.4%	2%	0.05%	0.4%	2%
0–24	8	5	8	10	8	8	7	7	6	8
24–48	6	4	6	8	6	6	6	6	7	7
48–72	6	6	6	8	6	16	6	10	56	66
72–96	6	11	12	12	65	142	52	147	125	154
96–120	6	98	137	138	100	170	148	152	157	175
120–144	6	127	202	202	102	140	180	159	138	175
144–168	8	173	202	219	112	128	176	135	127	130

[a] BACTEC 6B bottles were supplemented with the compounds listed at the final concentration indicated (by volume); each bottle was inoculated with 10^4 CFU of *M. furfur* and incubated at 35°C with shaking for 7 days.
[b] Growth index is proportional to $^{14}CO_2$ release. Mean of two bottles.

Table 1 shows the effect of various concentrations of Tween 80, Intralipid, and Liposyn on *M. furfur* (strain D) ^{14}C metabolism in BACTEC 6B bottles. Each lipid source supported active ^{14}C metabolism over the concentration range tested. Bottles supplemented with Liposyn at 0.4 or 2% yielded positive growth indices by 72 h, while Intralipid- and Tween 80-supplemented bottles yielded positive growth indices only at 96 and 120 h of incubation, respectively. Increase in CFU per milliliter paralled ^{14}C metabolism (Fig. 1). By 72 h, BACTEC 6B bottles supplemented with 2% Liposyn contained $>10^6$ CFU of *M. furfur* per ml, a concentration required to yield a positive BACTEC growth index. Both Intralipid- and Tween 80-supplemented bottles yielded $>10^6$ CFU/ml after an additional 24 to 48 h of incubation. Similar results were obtained with seven additional blood culture isolates of *M. furfur* (data not shown).

Table 2 shows the effect of inoculum size of *M. furfur* (strain D) on ^{14}C metabolism. Bottles supplemented with Intralipid and inoculated with 60 or 600 CFU yielded positive growth indices within 96 h; those inoculated with 6,000 CFU were positive by 72 h. Tween 80-supplemented bottles, however, did not yield positive growth indices when inoculated with 60 or 600 CFU/ml and incubated for 7 days; a positive growth index was achieved by 5 days of incubation in Tween 80 bottles inoculated with 6,000 CFU/ml

Table 3 shows the stability and recovery of *M. furfur* (strain D) from Isolator pediatric tubes. Organisms suspended in PBS or whole human blood and inoculated into Isolator tubes could be quantitatively recovered over an 8-h period at room temperature after plating onto GYP-S agar. In contrast, *M. furfur* was unstable in human blood inoculated into EDTA tubes. Similar results were obtained with seven other blood culture isolates of *M. furfur* and with strain D tested against six different sources of adult human volunteer blood (data not shown).

Simulated blood culture experiments were conducted to determine which blood culture system, the BACTEC 6B bottle or the Isolator pediatric tube, could recover *M. furfur* more reliably rapidly. The data in Fig. 2A show that less ^{14}C metabolism occurred in bottles to which human blood and Intralipid had been added, compared with bottles to which only Intralipid had been added. Positive growth index values (≥30) were not achieved in the blood- and Intralipid-supplemented bottles. In addition, the blood and Intralipid bottles developed a chocolate brown color with obvious

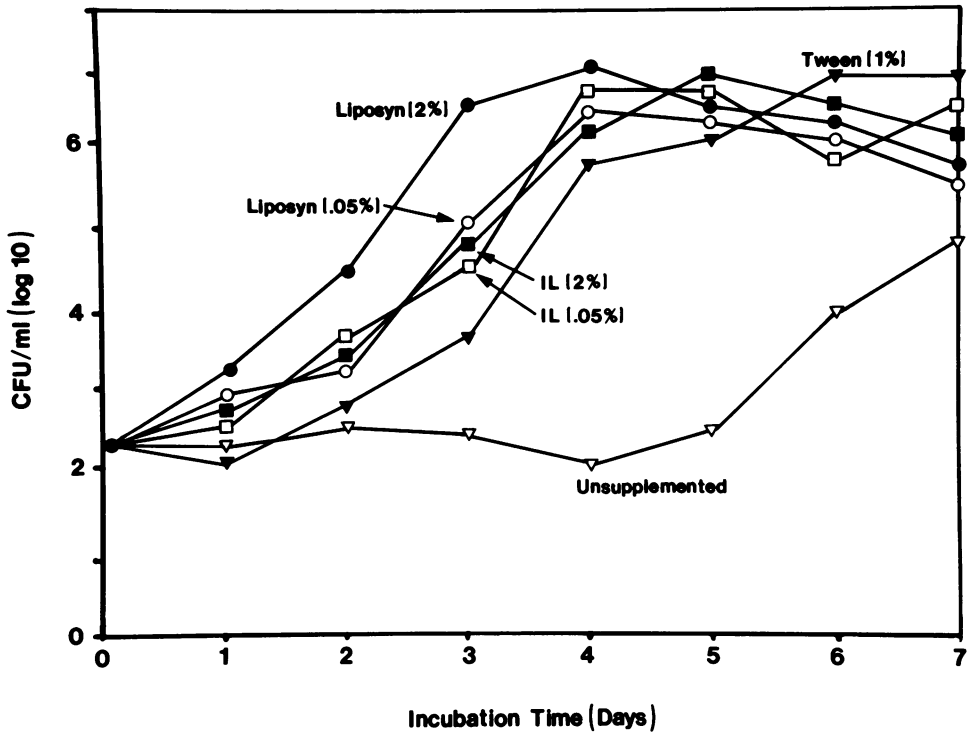

FIG. 1. Growth curves of *M. furfur* in supplemented BACTEC 6B broth. The following symbols represent the final concentration of each supplement in the bottles; ●, Liposyn (2%); ○, Liposyn (0.05%); ■, Intralipid (IL) (2%); □, Intralipid (0.05%); ▼, Tween 80 (1%); ▽, unsupplemented. All bottles were inoculated with 10^4 CFU of *M. furfur* at time zero and incubated at 35°C with shaking. Each data point represents the mean colony count from two bottles.

accumulation of sediment on the bottom. A reduction in viable organisms recovered by quantitative plate count of broth from the Intralipid- and blood-containing BACTEC bottles correlated with reduced ^{14}C metabolism (Fig. 2B); no organisms could be recovered by 48 h. Tween 80- and blood-containing bottles did support ^{14}C metabolism and growth of *M. furfur* (Fig. 2A and B); however, the data suggest some inhibition in Tween 80- and blood-containing bottles compared with bottles supplemented with the Tween 80. Similar results were obtained with six different sources of adult human volunteer blood and *M. furfur* inoculum sizes ranging from 100 to 10,000 CFU. On the other hand, *M. furfur* was quantitatively recovered from the Isolator pediatric tubes within 48 to 72 h after subculture, even when as few as 10 organisms were seeded into the tubes (data not shown).

TABLE 2. Effect of inoculum size of *M. furfur* on metabolism of ^{14}C-labeled substrates in supplemented BACTEC 6B broth[a]

Labeling period (h)	Growth index with varying inoculum size[b]					
	60 CFU		600 CFU		6,000 CFU	
	Tween 80	IL	Tween 80	IL	Tween 80	IL
0–24	7	7	8	7	8	7
24–48	6	6	5	6	7	7
48–72	7	7	6	11	6	54
72–96	6	50	6	133	6	132
96–120	7	149	6	178	110	155
120–144	6	164	6	122	179	128
144–168	6	163	5	95	130	101

[a] BACTEC 6B bottles were supplemented with 1% Tween 80 or Intralipid (IL) and inoculated with 60, 600, or 6,000 CFU of *M. furfur*; each bottle was incubated at 35°C with shaking for 7 days.

[b] Growth index is proportional to $^{14}CO_2$ release. Mean of two bottles.

DISCUSSION

We speculate that fat emulsions provide the fatty acid growth requirement of *M. furfur* for in vivo proliferation of this organism. *M. furfur* has been directly observed colonizing the lumenal wall of infected catheters by light as well as scanning electron microscopy (J. A. Novak, V. A. Ruppert, and M. A. Menegus, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, C348, p. 358; our unpublished observations). Blood cultures drawn from such catheters yield *M. furfur* when isolation media are supplemented with a long-chained fatty acid source. Peripheral blood cultures, however, have not commonly yielded *M. furfur*, suggesting that either fungemia occurs rarely or remains undetected for a variety of reasons. That at least two patients have been described with pulmonary vasculitis in association with abundant lipid

TABLE 3. Stability of *M. furfur* at room temperature in Isolator pediatric tubes[a]

Type of tube and fluid medium	CFU at various times (h) during incubation[b]				
	0	1	2	4	8
Isolator tube, PBS	85	82	72	72	66
Isolator tube, blood	60	47	66	44	67
EDTA tube, blood	63	34	31	25	18

[a] Human blood or PBS (0.01 M, pH 7.4) was inoculated with *M. furfur*, dispensed in 1.5-ml volumes into Isolator or EDTA tubes, and incubated at room temperature for 8 h. At indicated times, 0.1-ml samples were withdrawn and plated onto GYP-S agar, and colonies were counted after 3 days of incubation at 35°C.

[b] CFU/0.1 ml; mean of two tubes.

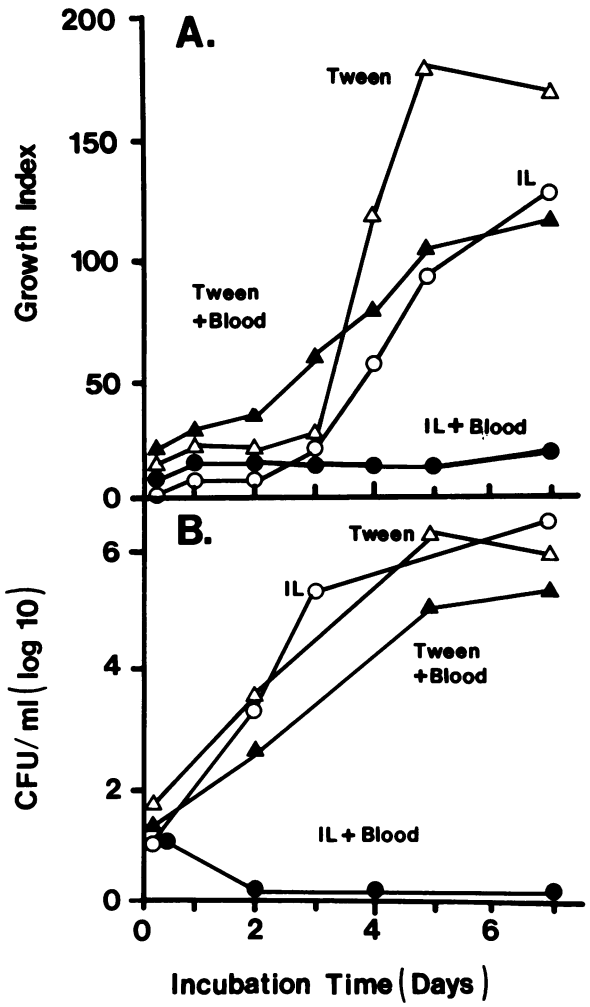

FIG. 2. Effect of whole human blood on *M. furfur* metabolism of ^{14}C-labeled substrates (A) and growth (B) in lipid-supplemented BACTEC 6B bottles. Cirles (○, ●) and triangles (△, ▲) represent bottles supplemented with 1% (vol/vol) Intralipid (IL) and Tween 80, respectively. Closed symbols represent bottles inoculated with 1.0 ml of blood; bottles without blood are indicated with open symbols. All bottles were inoculated with 1,000 CFU of *M. furfur* at time zero and incubated at 35°C with shaking. Each data point represents the mean growth index (A) or colony count (B) from two bottles.

deposits and *M. furfur* in pulmonary vessel walls suggests that a fungemia does occur (7, 13).

Commercially available blood culture broth media do not contain sufficient fatty acids to support the growth of *M. furfur*. However, we speculate that in some cases fat emulsions administered parenterally to patients for nutritional support may provide sufficient supplementation to blood culture medium to support limited in vitro growth of the organism. In this case, final concentration of the organism in broth medium probably does not exceed 10^5 or 10^6 CFU/ml and thus positive blood cultures may not be detected visually, microscopically, or radiometrically (15). Thus, to ensure detection of *M. furfur* in blood, a reliable and rapid alternative method is necessary.

In our study, lipid-supplemented BACTEC 6B bottles supported the ^{14}C metabolism and increase in CFU of *M. furfur*. However, positive growth index values were achieved only after several days of incubation, particularly with low initial inoculum of the organism. Moreover, in simulated blood culture experiments, the interaction of human blood and Intralipid produced a chocolate brown appearance and sediment in the BACTEC bottles and concomitant inhibition of ^{14}C metabolism. The brown sediment probably resulted from a lysis of erythrocytes and conversion of free hemoglobin in the medium to brown methemoglobin (1, 6). Erythrocyte lysis and inhibition of ^{14}C metabolism were observed over a concentration range of Intralipid and *M. furfur* inoculum sizes. Lysis was accelerated by shaking of the BACTEC bottles (data not shown), a procedure necessary to ensure the rapid radiometric detection of microorganisms.

Several published reports indicate that the Isolator lysis centrifugation-direct plating system is superior to broth systems for the recovery of yeasts from blood culture (2, 3). Our studies with *M. furfur* extend these observations to include this organism. *M. furfur* suspended in PBS-Triton or whole human blood was stable for up to 8 h in Isolator tubes and could be quantitatively recovered upon plating to GYP-S medium. A recent clinical case of *M. furfur* sepsis illustrates the utility of the Isolator system. A 9-week-old premature male was transferred to our hospital with a 2-day history of fever and thrombocytopenia and an unidentified nonviable yeast observed in blood culture bottles drawn at the referral hospital. Four Isolator blood cultures drawn through a Broviac catheter over a 6-day period in our hospital were positive within 24 to 48 h for *M. furfur*. Quantitative plate count on GYP-S agar indicated over 500 CFU/ml of blood. BACTEC blood cultures continued to be radiometrically negative, but the organism could be recovered by blind subculture from BACTEC 6B bottles onto GYP-S agar.

Based on these observations, we recommend that Isolator blood cultures be collected on patients receiving fat emulsion therapy and in whom sepsis is suspected. Blood can be directly plated onto GYP-S agar or, more conveniently, Sabouraud dextrose or blood agar to which a few drops of sterile olive oil have been added. The plates should be incubated at 35°C in air for a minimum of 4 days before discarding as negative. For those laboratories utilizing conventional or radiometric broth methods, a suitable alternative to Isolator may be blind Gram-stained smear and blind subculture of broth onto an agar medium capable of supporting the growth of *M. furfur*. The blood of patients receiving fat emulsion therapy probably contains sufficient amounts of lipid to support initial growth of *M. furfur* in blood culture bottles but insufficient amounts to produce macroscopic or radiometric positive blood cultures. At this point, we cannot recommend routine lipid supplementation of conventional or BACTEC blood culture bottles because of the possible in vitro inhibitory effect of blood and lipids on *Malassezia* spp. Moreover, the process of supplementing blood culture bottles increases the likelihood of introducing a contaminating organism and appears unnecessary. In any event, it is critical that both laboratory personnel and clinicians be aware of this "new" clinical entity so that appropriate procedures for isolation of *M. furfur* can be utilized in select patients.

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