Rapid and Direct Quantification of Viable *Candida* Species in Whole Blood by Use of Immunomagnetic Separation and Solid-Phase Cytometry[▽]

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***Candida* species are a common source of nosocomial bloodstream infections in critically ill patients. The sensitivity of the traditional diagnostic procedure based on blood culture is variable, and it usually takes 2 to 4 days before growth of *Candida* species is detected. We developed a 4-h method for the quantification of *Candida* species in blood, combining immunomagnetic separation (IMS) with solid-phase cytometry (SPC) using viability labeling. Additionally, *Candida albicans* cells could be identified in real time by using fluorescent *in situ* hybridization. By analysis of spiked blood samples, our method was shown to be sensitive and specific, with a low detection limit (1 cell/ml of blood). In a proof-of-concept study, we applied the IMS/SPC method to 16 clinical samples and compared it to traditional blood culture. Our method proved more sensitive than culture (seven samples were positive with IMS/SPC but negative with blood culture), and identification results were in agreement. The IMS/SPC data also suggest that mixed infections might occur frequently, as *C. albicans* and at least one other *Candida* species were found in five samples. Additionally, in two cases, high numbers of cells (175 to 480 cells/ml of blood) were associated with an endovascular source of infection.**

Candida species are a common source of nosocomial bloodstream infections in critically ill patients, with mortality rates exceeding 40%. Rapid detection and identification of *Candida albicans* and other *Candida* species could result in early initiation of adequate antifungal therapy, an important factor in reducing morbidity and mortality (3, 16).

At present, the gold standard for the detection of *Candida* species in the bloodstream is culture of blood samples. Although blood culture systems have evolved in recent years from manual to fully automated systems, the diagnostic sensitivity is still variable and differs greatly among studies, with 40 to 82% of blood culture bottles spiked with *Candida* or from patients with proven candidemia showing positive results (4, 6, 9, 10, 18). Possible explanations are the small numbers of *Candida* cells present in the blood during fungemia (10 to 25 cells per 10 ml of blood) (4, 10, 18), the use of growth media which are not optimal for fungal growth, and the presence of antimycotics in the blood (6). Additionally, it usually takes 2 to 4 days before growth of *Candida* species is detected in blood culture bottles (4, 10).

Several studies have shown good results by using PCR on DNA isolated from whole blood for the detection of candidemia (5, 13, 21, 22). However, one of the problems with PCR is the possibility of detecting DNA from dead and/or degrading yeast cells instead of living yeasts, leading to false-positive results (5, 13, 21, 22). Also, in many cases, only small sample

volumes can be used, or a long and cumbersome sample preparation is needed to reduce the influence of inhibitors present in blood (5, 13).

Another approach is to recover yeast cells by immunomagnetic separation (IMS) prior to further analysis. Magnetic beads coated with antibodies are used to capture the yeast cells present in the clinical sample, and separation occurs in a magnetic field (15). Although IMS has been used frequently for the recovery of specific microorganisms from different samples, the recovery rate is rather low (8, 15, 17). After separation of the cells from the sample, several analysis methods, such as plating, PCR, and solid-phase cytometry (SPC), can be used to quantify the number of microorganisms.

In SPC, the principles of epifluorescence microscopy and flow cytometry are combined. Microorganisms are retained on a membrane filter, fluorescently labeled, and automatically counted by a Chemsan RDI laser scanning device. Subsequently, the data for each fluorescent spot are analyzed by a computer to differentiate between fluorescent microorganisms and particles. Each retained spot can be inspected visually by an epifluorescence microscope (11, 20). Due to its low detection limit, speed, and possible use of taxonomic probes for identification, SPC has the potential to overcome the shortcomings of other methods for quantification of *Candida* species in blood samples (7, 14).

In the present study, a method for the rapid quantification of *Candida* species and identification of *C. albicans* in whole blood, based on IMS and SPC, is described. This method was optimized using spiked blood samples and subsequently used to analyze 16 blood samples from high-risk patients.

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TABLE 1. Overview of the fungi used for sensitivity and specificity testing and their reactivities with the capture antibody and the PNA probe used in the assay

Species	Strain designation ^a	Reactivity with antibody used for immunomagnetic capture	Reactivity with PNA probe used for identification of <i>C. albicans</i>
<i>Candida albicans</i>	ATCC MYA-682	+	+
	ATCC MYA-2876	+	+
	ATCC 10231	+	+
	IHEM 9559	+	+
	IHEM 10284	+	+
	MUCL 29903	+	+
	MUCL 29800	+	+
	MUCL 29919	+	+
	MUCL 29981	+	+
	MUCL 30112	+	+
<i>Candida dubliniensis</i>	NCYC 1467	+	+
	IHEM 14280	+	—
<i>Candida famata</i>	CI	+	—
<i>Candida glabrata</i>	MUCL 15664	+	—
	MUCL 29833	+	—
<i>Candida guilliermondii</i>	CI	+	—
<i>Candida inconspicua</i>	CI	+	—
<i>Candida kefyr</i>	CI	+	—
<i>Candida krusei</i>	IHEM 1796	+	—
<i>Candida lusitanae</i>	CI	+	—
<i>Candida maris</i>	CI	+	—
<i>Candida parapsilosis</i>	IHEM 3270	+	—
<i>Candida pseudotropicalis</i>	CI	+	—
<i>Candida tropicalis</i>	IHEM 4222	+	—
	IHEM 4225	+	—
	MUCL 29952	+	—
	MUCL 30002	+	—
<i>Aspergillus flavus</i>	IHEM 2700	—	—
<i>Aspergillus fumigatus</i>	IHEM 3768	—	—
<i>Cryptococcus neoformans</i>	IHEM 14513	—	—
<i>Fusarium graminearum</i>	IHEM 2994	—	—
<i>Fusarium oxysporum</i>	IHEM 14513	—	—
<i>Fusarium solani</i>	IHEM 6743	—	—
<i>Penicillium chrysogenum</i>	IHEM 3035	—	—
<i>Penicillium simplicissimum</i>	IHEM 1202	—	—
<i>Pseudallescheria boydii</i>	CBS 101.22	—	—
<i>Saccharomyces cerevisiae</i>	ATCC 9763	—	—
<i>Scedosporium prolificans</i>	CBS 467.74	—	—

^a ATCC, American Type Culture Collection; CBS, Centraalbureau voor Schimmelcultures (Netherlands); CI, clinical isolate from our own collection; IHEM, Instituut voor Hygiene en Epidemiologie (Belgium); MUCL, Mycothèque de l'Université Catholique de Louvain (Belgium); NCYC, National Collection of Yeast Cultures (United Kingdom).

MATERIALS AND METHODS

IMS/SPC method. Thirty microliters of polyclonal anti-*C. albicans* antibody conjugated with fluorescein isothiocyanate (FITC) (Acris Antibodies, Herford, Germany) and 30 µl monoclonal anti-FITC antibody bound to microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) were added to an EDTA-treated whole-blood sample (maximum volume, 15 ml). After incubation at room temperature for 1 h with head-to-tail rotation, the sample was loaded on a whole-blood column (Miltenyi Biotec). Prior to loading, the column was inserted into a QuadroMACS cell separator (Miltenyi Biotec) and prewashed with 3 ml of separation buffer (phosphate-buffered saline [PBS], 0.5% bovine serum albumin, and 2 mM EDTA, pH 7.2). Afterwards, the column was washed three times with 3 ml of separation buffer and removed from the magnetic field, and the yeasts were eluted with 5 ml of elution buffer (Miltenyi Biotec).

The eluate was filtered over a 2.0-µm Cycloblack-coated polyester membrane filter (AES-Chemunex, Ivry-sur-Seine, France), and filters were incubated at 55°C for 30 min with 100 µl of PNA^{Flow} reagent (AdvanDx, Vedbaek, Denmark) containing a FITC-conjugated peptide nucleic acid (PNA) probe specific for *Candida albicans* (19). Subsequently, filters were incubated with 100 µl Wash Buffer^{Flow} (AdvanDx) for 10 min at 55°C. Finally, tyramide signal amplification was used to obtain red fluorescent *C. albicans* cells. To this end, the filter was placed on top of 100 µl polyclonal anti-FITC antibody conjugated with horse-

radish peroxidase (AbD Serotec, Oxford, United Kingdom) (10 µg/ml PBS) for 30 min at 30°C. A tyramide Alexa Fluor derivative (Invitrogen, Leek, Netherlands) was then converted to its red fluorescent form by incubating the filter on 100 µl tyramide solution (stock solution diluted 1:50 in amplification buffer) for 30 min at 30°C. Between different steps, filters were rinsed three times with 1 ml PBS. Finally, all viable microorganisms were fluorescently labeled (green) by incubating the filters at 37°C for 1 h on a cellulose pad saturated with 600 µl ChemChrome V6 viability staining solution (stock solution diluted 1:100 in the labeling buffer ChemSol B2; AES-Chemunex). With this procedure, all fungal cells retained on the filter are labeled green.

The filters with labeled microorganisms were placed on a holder, on top of a support pad (AES-Chemunex) moistened with 100 µl of ChemSol B2 (AES-Chemunex), and scanned by a ChemScan RDI cytometer (AES-Chemunex). This solid-phase cytometer consists of an argon laser emitting light at 488 nm and two photomultiplier tubes to detect the fluorescent light emitted by the labeled cells. The produced signals are processed by a personal computer (PC), applying a series of software discriminants to differentiate valid signals associated with labeled *Candida* cells from other fluorescent particles. Subsequently, results are displayed as green spots on a membrane filter image in a primary scan map and, after software elimination of background, a secondary scan map (14). To confirm the results, visual inspection using an Olympus BX40 epifluorescence microscope

TABLE 2. Overview of characteristics of clinical samples included in the study and the results obtained after analysis with traditional blood culture and with IMS/SPC method

Patient	Blood culture result obtained before inclusion in study ^a	Sample collection date (mo-day-yr)	Analysis date (mo-day-yr)	Treatment prior to sample collection ^b	Results of analysis of clinical samples		
					Blood culture	No. of cells/ml of blood (IMS/SPC)	
						<i>C. albicans</i>	Other <i>Candida</i> spp.
A	—	06-16-09	06-16-09	—	Negative	3	3
B	<i>C. glabrata</i>	08-10-09	08-11-09	Fluconazole	Negative	0	2
C	<i>C. tropicalis</i>	08-11-09	08-12-09	Fluconazole	<i>C. tropicalis</i>	0	>175 ^c
	—	08-20-09	08-20-09	Fluconazole and anidulafungin	Negative	0	2
D	<i>C. albicans</i>	08-20-09	08-20-09	Fluconazole	Negative	3	0
E	<i>C. glabrata</i>	08-20-09	08-21-09	—	<i>C. glabrata</i>	3	4
F	<i>C. glabrata</i>	08-20-09	08-21-09	Fluconazole and caspofungin	<i>C. glabrata</i>	0	480
G	<i>C. albicans</i>	09-14-09	09-15-09	Fluconazole	Negative	0	0
H	<i>C. tropicalis</i>	09-14-09	09-15-09	Fluconazole	Negative	0	0
I	<i>C. albicans, C. glabrata</i>	09-14-09	09-15-09	Fluconazole	<i>C. albicans</i>	2	0
J	<i>C. glabrata</i>	10-06-09	10-07-09	—	<i>C. glabrata</i>	0	4
K	<i>C. glabrata</i>	10-07-09	10-08-09	—	Negative	6	3
L	—	10-07-09	10-08-09	—	Negative	0	0
M	<i>C. parapsilosis</i>	10-08-09	10-09-09	Fluconazole	Negative	1	6
N	<i>C. albicans</i>	12-04-09	12-05-09	Fluconazole	<i>C. albicans</i>	1	1
O	<i>C. krusei</i>	01-04-10	01-05-10	Caspofungin	Negative		

^a —, no positive blood culture was obtained prior to inclusion in this study.

^b —, patients did not receive treatment prior to inclusion in this study.

^c For this blood sample, 4 ml was filtered and more than 700 *Candida* cells were present on the filter, but a reliable quantification was impossible. Filtration of a smaller volume could have led to a more reliable quantification.

(Olympus, Tokyo, Japan) was performed. The filter holder was placed on a computer-driven moving stage in exactly the same position as in the Chemsan RDI machine. Subsequent highlighting of a green spot in the secondary scan map on the PC resulted in the direction of the microscope to the respective position on the membrane filter (2, 12), which allowed rapid visual inspection of all fluorescent spots. The red fluorescence of *C. albicans* cells can easily be observed using a 590-nm-cutoff emission filter (20).

Preparation of spiking solutions. In order to validate the IMS/SPC procedure, several experiments with spiked blood samples were carried out. An overview of the fungi used in these experiments is given in Table 1. These fungi were cultured overnight at 30°C in Sabouraud liquid medium, and serial dilutions were made in physiological saline. Subsequently, the number of cells was determined by SPC prior to spiking (20), and 1 ml of the obtained cell suspensions was used to spike the blood samples. For all spiking experiments, blood was freshly drawn from healthy volunteers, maintained at 4°C, and used in experiments the same day.

Evaluation of the sensitivity and specificity of the assay. For sensitivity testing, the recovery of all *Candida* species (27 strains) was determined by analysis of spiked blood samples (10 to 50 *Candida* cells/10 ml of blood) with IMS/SPC. The specificity of the antibody for immunomagnetic capture of *Candida* cells was tested by spiking blood with various fungi not belonging to the genus *Candida* (11 strains). Additionally, the specificity of the PNA probe for identification of *C. albicans* was examined using all other *Candida* strains (16 strains) (Table 1).

Determination of detection limit. In order to determine the detection limit of the IMS/SPC assay, a suspension of *C. albicans* ATCC MYA-2876 (76 cells/ml, as determined by SPC) was serially diluted (six times), and the numbers of cells present in the dilutions were also determined by SPC. Subsequently, blood samples from healthy volunteers were spiked with 1 ml of these solutions, and recovery with the IMS/SPC procedure was determined.

Comparison of IMS/SPC with blood culture, using spiked blood. In order to compare the IMS/SPC method with conventional blood culture, five whole-blood samples (10 ml) were obtained from 10 healthy volunteers. One unspiked sample per healthy volunteer was used as a negative control and was analyzed with the IMS/SPC assay simultaneously with the other samples. Two samples per volunteer were spiked with *C. albicans* ATCC MYA-2876, and the other two were spiked with *C. dubliniensis* IHME 14280, *C. glabrata* MUCL 29833, *C. krusei* IHME 1795, *C. parapsilosis* IHME 3270, or *C. tropicalis* MUCL 29952. The whole-blood samples were spiked with 1 to 100 *Candida* cells. Two spiked whole-blood samples were analyzed immediately by IMS/SPC, and two were analyzed by conventional blood culture. To that end, the last two spiked blood samples were aseptically introduced into a BacT/Alert FA blood culture bottle

(bioMérieux, Marcy l'Etoile, France), and the bottle was incubated at 37°C. The change of color of the indicator was monitored visually.

Storage of blood samples. In order to investigate if storage of the blood samples at 4°C has an influence on the viability of *Candida* cells, analysis was performed immediately and 3, 6, 9, 16, and 24 h after spiking. To that end, two times six blood samples (5 ml) from a healthy volunteer were spiked with 24 or 76 *C. albicans* ATCC MYA-2876 cells (cell number determined by SPC) and stored at 4°C until analysis.

Proof of concept by analysis of clinical samples. Finally, 16 samples from 15 patients with candidemia or suspected candidemia were analyzed (Table 2). This research was approved by the ethical committee of University Hospitals Leuven (Belgium) (study number S51459). Blood samples were stored at 4°C until analysis, and all samples were analyzed within 24 h of collection (Table 2). An equal aliquot of the blood sample was analyzed both by the IMS/SPC procedure and by using a BacT/Alert FA blood culture bottle that was monitored visually. If the blood culture was positive, a subculture on Chromagar (BD) was made, and subsequent identification of all yeasts recovered was performed using an API-20C AUX system (bioMérieux).

RESULTS

Evaluation of the sensitivity and specificity of the assay. The sensitivity of the assay was confirmed, as the average recovery ($\pm$ standard deviation) for all *Candida* strains tested was 114% ($\pm 20\%$) ($n = 27$) after IMS/SPC analysis. The method proved highly specific, and only cells belonging to *Candida* species were retained using the IMS procedure (Table 1). The specificity of the PNA probe was confirmed, as only *C. albicans* cells yielded red fluorescence (Table 1).

Determination of detection limit. A linear correlation between the numbers of cells (ranging from 0 to 76, as determined with SPC prior to spiking) in the spiking solutions and the numbers of cells found in the blood samples supplemented with this solution was obtained (data not shown). The average recovery ($\pm$ standard deviation) was 116% ($\pm 18\%$), and the detection limit was one cell per 10 ml of blood.

FIG. 1. Numbers of *Candida* cells in spiking solutions (white bars) compared to the numbers obtained after application of the IMS/SPC procedure to spiked blood samples from 10 healthy volunteers (gray bars).

Comparison of IMS/SPC with blood culture, using spiked blood. In order to compare the results obtained with IMS/SPC and conventional blood culture, we analyzed spiked blood samples from 10 healthy volunteers in parallel. The average recovery of *Candida* cells with the IMS/SPC procedure was $95\% \pm 29\%$ ($n = 20$) and was independent of the species. The absolute differences in numbers of cells in the spiking solutions and blood samples analyzed by IMS/SPC were very small (Fig. 1). No *Candida* cells were detected in any of the negative-control samples. A visual color change of the CO₂ sensor in all blood culture bottles was seen within 25 h.

Storage of blood samples. We also determined whether storage (4°C) of blood samples had an influence on the number of yeast cells detected and could not observe a reduction in the number of cells when the sample was analyzed within 9 h of storage (Fig. 2). However, a marked decline (as high as 75%) in the number of cells (independent of the absolute number) was found after 16 h of storage (Fig. 2).

Proof of concept by analysis of clinical samples. Sixteen clinical samples were analyzed with IMS/SPC and blood culture. Three samples were negative with both methods, six were

FIG. 2. Numbers of *C. albicans* cells obtained with the IMS/SPC procedure for two spiked blood samples from one healthy volunteer (triangles and squares), determined at different time points during storage of the samples at 4°C.

positive with both methods, and seven were positive with IMS/SPC but negative with blood culture. For some samples, visual and automated detection of a change in color of the indicator on the bottom of the blood culture bottle was performed, and the same results were obtained (data not shown). Additionally, upon subculture, no *Candida* cells were isolated from any of the negative blood culture bottles.

Absolute counts obtained with IMS/SPC ranged from 2 to 480 *Candida* cells per ml (Table 2). For two patients, extremely high numbers of *Candida* cells were found in the blood sample (>175/ml for patient C and 480/ml for patient F). In both cases, a contaminated catheter was removed after collection of the sample. For patient C, a follow-up sample was obtained 9 days after removal of the catheter and the start of anidulafungin treatment. This sample contained fewer cells (2 cells/ml by IMS/SPC analysis) and was no longer positive by blood culture (Table 2).

The differentiation between *C. albicans* and other *Candida* species obtained with IMS/SPC was in agreement with the results obtained with culture for the six samples which were positive by both methods (Table 2). For the clinical samples that were negative by blood culture in this study but positive by IMS/SPC, the differentiation between *C. albicans* and other *Candida* species obtained with the IMS/SPC assay was in agreement with the identity of the isolate recovered before inclusion in the present study. Patients A, E, K, M, and N presented with mixed infections according to the IMS/SPC analysis, while this was not observed using the culture-based method (Table 1). For patient I, *C. glabrata* and *C. albicans* were isolated from a blood culture 3 days before inclusion in our study. With IMS/SPC, only *C. albicans* cells were found in the sample.

DISCUSSION

We report the development of a 4-h method for the quantification of *Candida* species in blood, based on IMS and SPC, using viability staining and fluorescent *in situ* hybridization (FISH) to label all *Candida* species and *C. albicans*, respectively. Analysis of spiked blood samples indicated that our method is specific, has a low detection limit, and allows accurate quantification.

Using spiked blood samples, we did not observe an improved diagnostic sensitivity with the IMS/SPC method. Unlike the case in numerous reports in literature, all blood cultures of spiked samples were positive (4, 10, 18). This can be explained by the use of laboratory strains to spike the blood cultures. However, seven clinical samples were positive with IMS/SPC but negative with blood culture. The latter observation clearly indicates that the IMS/SPC assay has a higher diagnostic sensitivity than blood culture. For four of the samples for which blood culture was negative but IMS/SPC was positive, treatment with antifungal drugs prior to collection might be an explanation for the divergent results (Table 2).

We observed that analysis of blood samples within 9 h of sample collection is recommended due to a reduction in the number of yeast cells recovered after longer storage. Nevertheless, even for samples that were analyzed more than 9 h after sampling (Table 2), we were able to quantify the number of *Candida* cells present, although it is likely that the number

observed was an underestimation of the number of cells actually present at the time of sampling.

The IMS/SPC method allows not only the rapid diagnosis of candidemia but also the accurate quantification of *Candida* cells. This might help in assessing the response to antifungal therapy or may lead to the decision to remove intravascular devices (18). Our data suggest that a large number of *Candida* cells present in a blood sample is associated with a contaminated intravascular device (18), although only two samples were investigated and further research is required to support this hypothesis.

The present study also demonstrates that mixed infections might occur more frequently than generally assumed on the basis of culture-based techniques. While in 5 of the 16 clinical samples *C. albicans* and at least one other *Candida* species were found by use of the IMS/SPC procedure, not a single mixed infection was identified by use of blood culture.

Only one previous study has used IMS for the recovery of *Candida* cells from spiked blood (1). However, this study used culture after capture of the cells, and therefore results were obtained only 1 day before results could normally be obtained with blood culture. Additionally, identification of the *Candida* species was possible only after culture, and some species were not captured by the antibody. Finally, the low recovery rate (11 to 43%) observed for spiked blood samples was a crucial problem limiting the use of the method for the analysis of clinical samples containing small numbers of *Candida* cells (1).

Several research groups have obtained good results by PCR on DNA isolated from blood for the diagnosis of candidemia (5, 13, 22). The time to result for a commercial real-time PCR assay (SeptiFast; Roche) is 6 h, which is comparable to that for our method. However, one of the problems of PCR methods is the possible detection of DNA from dead and/or degrading cells instead of living yeasts. To avoid this, a labeling step using the viability stain ChemChrome V6 was included in the SPC procedure described in the present study.

PNA FISH methods are increasingly being used in hospitals for the identification of *Candida* species after the detection of yeasts in blood culture bottles. In our study, we used a commercially available PNA probe for *C. albicans* identification, but by using IMS and SPC, we could omit the time-consuming culture step (19). The implementation of additional PNA probes allowing the identification of other *Candida* species might further improve our method.

In conclusion, our study provides a proof of principle for the diagnostic potential of IMS combined with SPC for the detection of *Candida* cells in whole blood. In its current form, the method may be too laborious for implementation in a routine diagnostic laboratory. Further automation of the technique combined with an expansion of the panel of pathogens that can be detected and identified in blood could make this technique more attractive for diagnostic laboratories in the future.

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