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Short Communication

An extraction method of positive blood cultures for direct identification of *Candida* species by Vitek MS matrix-assisted laser desorption ionization time of flight mass spectrometry

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Candida spp. are an important cause of nosocomial bloodstream infections. Currently, complete identification of yeasts with conventional methods takes several days. We report here the first evaluation of an extraction method associated with the Vitek MS matrix-assisted laser desorption ionization time of flight mass spectrometry for direct identification of *Candida* species from positive blood cultures. We evaluated this protocol with blood cultures that were inoculated with reference and routine isolates (eight reference strains, 30 patients isolates and six mixed cultures containing two strains of different *Candida* species), or from patients with candidemia (28 isolates). This method performed extremely well (97% correct identification) with blood cultures of single *Candida* spp. and significantly reduced the time of diagnosis. Nevertheless, subculture remains indispensable to test fungal resistance and to detect mixed infections.

Keywords *Candida*, blood culture, extraction, identification, MALDI-TOF

Introduction

Candidemia is the fourth most common nosocomial bloodstream infection, causing significant mortality and the incidence of such invasive fungal infections is increasing [1,2]. Those at risk for these infections are immunosuppressed patients, have undergone complex abdominal surgery or were admitted into intensive care units. Early and appropriate treatment is essential to successfully control these fungal diseases [3]. Currently, using conventional laboratory techniques, complete identification of a yeast from a positive blood culture requires at least 48 hours, and sometimes up to several days. New technologies like matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)-based systems are promising alternatives to save time in identifying yeasts and quickly initiate antifungal therapy. Up until now, the Vitek MS system has only been evaluated with colonies from Sabouraud agar plates [4].

The use of MALDI-TOF MS directly on positive clinical blood culture samples might present some limitations. Indeed, human or culture broth proteins, that can interfere with the spectra of microorganisms, may limit yeast identification. The extraction procedure of the yeasts in culture is therefore a critical step to remove these unwanted proteins without altering fungal ones.

We report here the first evaluation of an extraction method associated with the Vitek MS system (bioMérieux,

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France) for the direct identification of *Candida* spp. from fungal positive Bactec Mycosis IC/F blood cultures (Becton Dickinson, France). The aims of the current study were: (i) to develop and optimize a protocol for the extraction of fungal proteins from the blood culture broth to allow direct identification of yeast species by the Vitek MS system, (ii) to evaluate this extraction with blood cultures inoculated with routine isolates, and (iii) to test this extraction procedure with under routine clinical conditions, on patients' positive blood cultures.

Materials and methods

Strains

The following eight reference strains were used to develop the extraction protocol: *Candida albicans* ATCC 9002 and 14053, *Candida parapsilosis* ATCC 22019 and 7330, *Candida glabrata* ATCC MYA-2950, *Candida krusei* ATCC 6258 and 14243, *Candida dubliniensis* ATCC MYA-646. Thirty different *Candida* isolates recovered from clinical samples that had been previously identified by routine laboratory techniques were then used to test this extraction protocol. Routine identification techniques employed to identify these isolates were: *C. albicans* was identified using CHROMagar chromogenic medium (Becton Dickinson, Heidebelberg, Germany) associated with the formation of chlamydospores on potato/carrot/bile agar (Bio-Rad, Marnes-La-Coquette, France); *C. dubliniensis* and *C. krusei* identification was achieved by the rapid latex agglutination test (Fumouze Diagnostics, Levallois-Perret, France); and ID32 C (bioMérieux, la Balme, France) was used for all other yeasts. Following the development of our method, it was employed with all patient positive blood cultures during a period of four months in the University Hospital of Toulouse (France). To simulate infections caused by mixed *Candida* species, we used three clinical isolates, one each of *C. albicans*, *C. glabrata* and *C. krusei* that were previously identified by routine laboratory techniques.

Artificial inoculation of blood cultures

Bactec Mycosis IC/F blood cultures (Becton Dickinson, France) were filled with 10 ml of blood from healthy volunteers (Centre de Transfusion Sanguine). Each bottle was inoculated with 100 µl of a suspension adjusted to 10^4 yeasts/ml in 0.9% sodium chloride as previously described [5] and then incubated at 35°C with agitation in a Bactec 9240 automat (Becton Dickinson, France) until visible growth was detected after more than 24 h and less than 72 h of inoculation. This range corresponds to the time interval during which most clinical blood cultures appear positive in actual cases of candidemia. For control tests, each bottle was inoculated with 10 ml of blood from healthy volunteers and 100 µl of saline serum. In order to evaluate the performance of the extraction protocol to detect mixed *Candida* species infections, *C. albicans* was mixed with *C. glabrata* or *C. krusei* in the proportions 50:50%, 25:75% or 75:25% and then inoculated to blood cultures, with two blood cultures for each mixture.

Sample extraction for proteomic analysis

The extraction method used in this study was an adaptation of protocol described by Marinach-Patrice *et al*. [5]. Two milliliters of the positive blood culture were centrifuged at 9600 g (2 min). The supernatant was discarded and the pellet washed with 1.5 ml of sterile distilled water. Following a further centrifugation (9600 g, 2 min), the supernatant was again discarded and the pellet re-suspended with 1.5 ml of a basic solution of sodium dodecylsulfate (SDS 0.1%, $NaHCO_3$ 0.015M) and incubated for 10 min at 37°C (heating block). The mixture was then centrifuged (9600 g, 2 min) and the pellet washed with 1.5 ml of sterile distilled water and re-centrifuged (9600 g, 2 min). The pellet was washed with 1.5 ml of sterile distilled water and centrifuged (9600 g, 2 min) for a second time before being suspended in 900 µl of absolute ethanol and 300 µl sterile distilled water. After centrifuging (9600 g, 2 min), the supernatant was discarded and 50 µl of pure acetonitrile and 50 µl of formic acid were added and vortexed thoroughly.

MALDI-TOF mass spectrometry analysis

One and a half microliters of extract were deposited in duplicate on the target. When dry, the spot was covered with 1 µl of ready-to-use α-cyano-4-hydrocinnamic acid (CHCA) matrix (bioMérieux, France). As a control for the extraction, one colony from a subculture on a Sabouraud agar plate was also applied in duplicate onto a target as described by Iriart *et al*. [4]. Analyses were performed on the Vitek MS instrument in a positive linear mode, according to the manufacturer instructions. Species identification was carried out using the bioMérieux spectrum classifier algorithm and the spectral database MS-ID version 1.

Analysis of results

The identification was considered as correct ('concordant identification') if at least one of the duplicate spots gave the same identification as that obtained by routine laboratory techniques, provided there were no major discordances of identification between the duplicates. There was 'no identification' if the Vitek MS database provided no results or a low discrimination (between the

correct species and another species) which did not allow precise species identification. We concluded 'discordance' if a species was wrongly identified in the place of another.

Results

Optimized extraction protocol

The protocol for the extraction of fungal proteins was optimized using simulated positive blood cultures obtained by the inoculation of reference strains of eight *Candida* species. The Marinach-Patrice *et al*. [5] protocol was refined by adding an incubation step of 10 min at 37°C, which significantly increased performances, particularly for blood cultures that were slow in being detected positive by the Bactec automat (> five days). With this method, all eight *Candida* reference strains were correctly identified on all spots (Table 1).

Evaluation of the extraction protocol on simulated positive blood cultures

In a second series of experiments, the same extraction method was evaluated on simulated positive blood cultures inoculated with patients' isolates. Thirty blood cultures were inoculated with three different isolates of 10 *Candida* species recovered from clinical samples (Table 1). One hundred percent of the 10 species were correctly identified except for one of the duplicate spots of two *C. dubliniensis* isolates due to a low discrimination with *C. albicans* on one spot. This low discrimination was also observed on the two spots of *C. dubliniensis* obtained after subculture. All the six negative control blood cultures gave 'no identification', as expected.

Regardless of the species mixed with *C. albicans* or the ratio of the inocula, only *C. albicans* was identified in all 24 spots (12 simulated mixes in duplicate).

Evaluation of the extraction protocol on positive blood cultures from patients with candidemia

The last part of the study consisted of evaluating positive blood cultures from patients which included 28 *Candida* positive cultures (Table 1), one of *Fusarium*, one with bacteria and one false positive. Among the 28 *Candida* blood cultures, 26 were correctly identified (92.9%). All 16 *C. albicans* and three *C. guillermondii* were correctly identified and among the eight *C. glabrata* tested, only one

Table 1 Identification of *Candida* spp. by Vitek MS after direct extraction of blood cultures.

Species	No. of different strains	Vitek MS identification results	Concordant identification [no. (%)]	No identification [no. (%)]	Discordance [no. (%)]
Reference strains					
C. albicans	2	*C. albicans*	2 (100)		
C. parapsilosis	2	*C. parapsilosis*	2 (100)		
C. glabrata	1	*C. glabrata*	1 (100)		
C. krusei	2	*C. krusei*	2 (100)		
C. dubliniensis	1	*C. dubliniensis*	1 (100)		
Total	8		8 (100)	0 (0)	0 (0)
Isolates from patients					
C. albicans	3	*C. albicans*	3 (100)		
C. parapsilosis	3	*C. parapsilosis*	3 (100)		
C. glabrata	3	*C. glabrata*	3 (100)		
C. krusei	3	*C. krusei*	3 (100)		
C. dubliniensis	3	*C. dubliniensis/C. albicans*∗	3 (100)∗		
C. tropicalis	3	*C. tropicalis*	3 (100)		
C. kefyr	3	*C. kefyr*	3 (100)		
C. lusitaniae	3	*C. lusitaniae*	3 (100)		
C. guilliermondii	3	*C. guilliermondii*	3 (100)		
C. inconspicua	3	*C. inconspicua*	3 (100)		
Total	30		30 (100)	0 (0)	0 (0)
Candidemia					
C. albicans	16	*C. albicans*	16 (100)		
C. glabrata	8	*C. glabrata/No identification*	7 (87.5)	1 (12.5)	
C. tropicalis	1	*No identification*		1 (100)	
C. guilliermondii	3	*C. guilliermondii*	3 (100)		
Total	28		26 (92.9)	2 (7.1)	0 (0)
Total (all strains)	66		64 (97.0)	2 (3.0)	0 (0)

∗Low discrimination between *C. dubliniensis* and *C. albicans* observed for the identification after subculture and after direct extraction (for two different strains on one of the two duplicate spots).

isolate was not identified (87.5% correct identification). One blood culture with *C. tropicalis* was tested but gave 'no identification'. Blood cultures with *Fusarium*, bacteria and false positive blood culture gave 'no identification', as expected.

Discussion

A total of 97.0% of all isolates included in this study were correctly identified (Table 1), which showed that the extraction protocol used with the Vitek MS system performed well with blood samples.

This extraction protocol was established using the Bactec Mycosis IC/F blood cultures (Becton Dickinson, France) because they do not contain charcoal, a component that is not compatible with MALDI-TOF systems. We added an incubation step (10 min at 37°C), to the Marinach-Patrice *et al*. protocol [5], which significantly improved the performance, particularly for the blood cultures that the Bactec automat was slow to detect (> five days) as positive without significantly increasing the time of analysis (< 1 h for the complete identification). This extraction protocol is inexpensive (less than two Euros per sample), quick and can be used with the classic configuration of the bioMérieux Vitek MS system without modification of the automat or of the database. To date, few studies have specifically tested the application of the MALDI-TOF MS directly on candidemia samples. All earlier studies were carried out with the Bruker system, but the results obtained with it were comparable to those found in this investigation of the Vitek MS [5–7].

Only 3% of all blood cultures were not identified which involved one of *C. glabrata* and one *C. tropicalis* from patients with candidemia who received antifungal therapy when the blood samples were collected. In the cases of the blood culture inoculated with non-clinical samples of known species, two isolates of *C. dublinensis* were only identified on one spot. As noted on several occasions with the MALDI-TOF technique, there is a low discrimination between *C. dubliniensis* and *C. albicans* for the other duplicate spots [8]. As this problem was also observed on the two spots of *C. dublinienis* obtained after subculture, this misidentification is not related to the extraction method. All non-*Candida* cultures gave 'no identification' as expected, showing the good specificity of the method.

As described previously by others, one of the difficulties with the MALDI-TOF MS approach of direct identification of *Candida* spp. from blood cultures is the possible occurrence of polymicrobial bloodstream infections [6]. Surprisingly, and in contrast to the results reported by Spanu *et al*. [6], in our hands, a partial identification was obtained for all mixed *Candida* species blood cultures. Fortunately, polymicrobial bloodstream infections are relatively rare in clinical practice (24% yeast and bacteria and 3% two yeasts) [1,9]. As the Vitek MS was unable to identify two yeasts in the same vial, subcultures remain indispensable and results should be delivered as 'presumptive identification', until the isolates is grown in subculture. Moreover, even if some approaches seem to be promising to evaluate the antifungal resistance directly by mass spectrometry, the subculture is also still necessary to determine the minimal inhibitory concentrations (MIC) of each strain [10].

In conclusion, this easy extraction method combined with the Vitek MS system constitutes a powerful alternative for the identification of *Candida* spp. directly from positive blood cultures. This method is easily adaptable to use in clinical routine. Even if these results from limited specimens constitutes a preliminary study, which should be confirmed by a larger scale investigation, this method may reduce the delay in diagnosis and allow the establishment of an earlier, more appropriate antifungal therapy for patients.

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