Eur J Clin Microbiol Infect Dis (2014) 33:1247–1252
DOI 10.1007/s10096-014-2069-z

ARTICLE

Assessment of caspofungin susceptibility of *Candida glabrata* by the Etest®, CLSI, and EUCAST methods, and detection of *FKS1* and *FKS2* mutations

N. Bourgeois · C. Laurens · S. Bertout · Y. Balard · D. Krasteva · P. Rispail · L. Lachaud

Received: 7 October 2013 / Accepted: 24 January 2014 / Published online: 13 February 2014
© Springer-Verlag Berlin Heidelberg 2014

Abstract *Candida glabrata* has emerged as a major pathogen in invasive candidiasis in recent years. Currently, guidelines for invasive candidiasis treatment recommend fluconazole or an echinocandin as the first-line therapy. Nevertheless, the resistance of *Candida glabrata* to echinocandin is an emerging problem and has been partly associated with mutations in the *FKS1* and *FKS2* genes. The Etest® is an appropriate method for determining antifungal susceptibility in emergency routine diagnosis. In this work, we evaluated the reliability of the Etest® in comparison with the two reference broth microdilution methods, Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST), to assess the caspofungin resistance of 193 isolates of *Candida glabrata*. The interpretation of minimum inhibitory concentration (MIC) values was also discussed according to different breakpoints. Moreover, *FKS1* and *FKS2* mutations were investigated for isolates with high MICs. Our results showed that the MIC_{50} value was similar to the MIC_{90} value for each method. The Etest® method showed the lowest MIC values, whereas EUCAST presented the highest. Categorical agreement between the Etest® and CLSI methods was 100 % and 36 % using the breakpoints proposed by Arendrup et al. (Antimicrob Agents Chemother 56(7):3965–3968, 2012) and Pfaller et al. (Int J Antimicrob Agents 38(1):65–69, 2011), respectively. Two isolates showed high MIC values with the three methods and both presented *FKS2* mutations. A novel *FKS2* mutation was also reported for one isolate. Future epidemiological studies should also evaluate the reliability of the Etest® to detect echinocandin resistance, as it remains a routine method.

N. Bourgeois
Laboratoire de Parasitologie-Mycologie, CHU de Nîmes et CHU de Montpellier, Montpellier, France

C. Laurens
Laboratoire de Bactériologie, CHU de Montpellier, Montpellier, France

S. Bertout · D. Krasteva
Laboratoire de Parasitologie-Mycologie, Université Montpellier 1/Faculté de Pharmacie, Montpellier, France

Y. Balard · P. Rispail
Laboratoire de Parasitologie-Mycologie, CHU de Montpellier, Université Montpellier 1/Faculté de Médecine, Montpellier, France

L. Lachaud (✉)
Laboratoire de Parasitologie-Mycologie, Centre Hospitalier Universitaire Caremeau, Place du Pr Debré, 30029 Nîmes cedex 9, France
e-mail: laurence.lachaud@univ-montp1.fr

Introduction

It is commonly admitted that the implication of *Candida glabrata* in invasive fungal infections has increased significantly [3]. According to antifungal therapy guidelines, the first-line therapy of invasive candidiasis is fluconazole or an echinocandin [4–6], a broad-spectrum anti-*Candida* molecule, in particular for *Candida glabrata*, which has a high rate of resistance to azoles [2]. Echinocandins inhibit the synthesis of glucan, a major structural polysaccharide of the fungal cell wall, via the inhibition of 1-3 beta-D-glucan synthase. However, resistance to these molecules is an emerging problem for this *Candida* species, as detailed in recent case reports and surveillance studies [2, 7]. Echinocandin resistance is related more often to *Candida albicans* and *Candida glabrata*, even if other species can be affected. Determination of the in vitro minimum inhibitory concentration (MIC) may allow the detection of resistance and then to avoid therapeutic failure in patients receiving such treatment. Among the mechanisms of resistance to caspofungin, chitin upregulation [8] or mutations of the *FKS* gene encoding for the subunits of the beta-D-glucan synthase have been reported [9, 10]. Thus, mutations

in *FKS1* for *Candida albicans*, *Candida glabrata*, *Candida krusei*, and *Candida tropicalis*, and only *FKS2* for *Candida glabrata*, have been associated with resistance, for which the level is linked to the location of the mutation in hotspot regions [10–12].

For several years, yeast antifungal susceptibility tests have been widely used in routine practice to assess the decision-making process, the probability of therapeutic failure in case of invasive candidiasis, and also epidemiologic surveillance in hospital. Two reference broth microdilution methods are available for the antifungal susceptibility testing of yeasts: the Clinical and Laboratory Standards Institute (CLSI) method and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) method. Nevertheless, these reference methods are too cumbersome and time consuming to be used in routine practice. Commercial kits, such as the disk diffusion or Etest® methods, more appropriate for routine diagnosis, were developed to determine the MIC values. Nevertheless, commercial kits still need to be evaluated and compared to reference methods to prove the reliability of MIC results.

The aim of this study was to evaluate the reliability of the Etest® method routinely used in our laboratories to test the susceptibility of *Candida glabrata* to caspofungin. For that purpose, 193 isolates of *Candida glabrata* were selected and the Etest® MIC values were compared to the MIC values obtained with both the CLSI and EUCAST reference methods. Moreover, the detection of mutations of *FKS2* and *FKS1* was performed for isolates that presented high MIC values with at least one of the three methods.

Materials and methods

Candida isolates

All of the 193 *Candida glabrata* isolates used in this study were from patients hospitalized in Montpellier or Nîmes University hospitals between May 2009 and October 2010. Of these isolates, 29 were from blood cultures, 17 from abdominal sterile sites, 25 from deep pulmonary samples, 113 from urine samples, and nine were from different sites in multicolonized patients.

Mycological identification

The identification of *Candida glabrata* was performed by using ID 32C (bioMérieux) or Vitek 2 YST ID Card (bioMérieux).

Antifungals and methods

Caspofungin MICs were determined by three methods: the CLSI method according to the CLSI M27-A3 document's recommendations approved in April 2008 (CLSI M27-A3, 2008), the EUCAST method [13], and the Etest® method (bioMérieux, France), according to the manufacturer's instructions (inoculum 0.5 McF, medium RPMI1640 2 % glucose agar, incubation time 24/48 h, temperature 35 ± 2 °C, and reading at 80 % of inhibition). Caspofungin powder was provided by MSD and the same lot of pure caspofungin substance was used for both the CLSI and EUCAST methods.

Quality control

The three methods were validated using three ATCC quality control strains, ATCC 90028 (*Candida albicans*), ATCC 22019 (*Candida parapsilosis*), and ATCC 6258 (*Candida krusei*), as recommended. ATCC control isolates were run in each set of experiments for the three methods.

Breakpoints

The CLSI interpretative breakpoints used for *Candida glabrata* were those initially proposed by Pfaller et al. [2] (susceptible ≤0.12 mg/L, intermediate=0.25 mg/L, resistant ≥0.5 mg/L) and slightly modified by Arendrup et al. [1] (susceptible ≤0.5 mg/L). For the Etest®, we used breakpoints recommended by the manufacturer for all *Candida* species: susceptible ≤2 mg/L and not-susceptible >2 mg/L, and also those proposed above for the CLSI method; for the EUCAST method, there is no recommended breakpoint for *Candida glabrata*.

Statistics

A two-dilution MIC difference was required to calculate the essential agreement (EA) between two methods, or between the 24- and 48-h end point readings. To compare methods, EAs were calculated at the 48-h end point for the Etest® and CLSI methods. Interpretative breakpoints were used to examine categorical agreement (CA). Discrepancies were categorized into three groups: (i) very major errors (VME; isolates interpreted as susceptible with the Etest® and resistant with the CLSI method), (ii) major errors (ME; the isolate was resistant by the Etest® and susceptible by the CLSI method), and (iii) minor errors (mE; i.e., either susceptible or resistant by one method and intermediate by the other).

Detection of *FKS1* and *FKS2* mutations

FKS1 and *FKS2* mutations were determined for a total of 18 strains of *Candida glabrata*. After culture on a Sabouraud chloramphenicol agar plate (24 h, 35 °C), *Candida glabrata* genomic DNA was extracted from yeast according to the Macherey-Nagel NucleoSpin QuickPure protocol. The hotspot regions of the *FKS1* and *FKS2* genes were amplified

Table 1 MIC_{50} and MIC_{90} values obtained for caspofungin with the EUCAST, CLSI (24 h and 48 h), and Etest® (24 h and 48 h) methods, and essential agreement between the 24- and 48-h readings for the Etest® and CLSI methods

	Method	MIC (mg/L)			MIC (mg/L)			EA[a]
		24 h			48 h			
		Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	
Caspofungin	EUCAST	0.125–16	0.5	0.5	–	–	–	–
	CLSI	0.03–16	0.25	0.25	0.03–16	0.25	0.25	95.9 %
	Etest	0.032–32	0.094	0.125	0.047–32	0.125	0.125	100 %

[a] Essential agreement between the MIC values obtained at 24 h and 48 h for the same method

as previously described [14]. Amplification products were sequenced by Cogenics (London, UK). The DNA sequences were analyzed with BLAST® (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

The caspofungin MICs of 193 *Candida glabrata* strains were assessed by the EUCAST, CLSI, and Etest® methods. Table 1 shows the MIC_{50} and MIC_{90} values, as well as the concordance between the 24- and 48-h readings, for each method. Our results showed that the MIC_{50} value was similar to the MIC_{90} value for each method. The Etest® showed the lowest values ($MIC_{50\text{-}90}$=0.125), followed by the CLSI method ($MIC_{50\text{-}90}$=0.25), whereas the EUCAST method showed the highest value ($MIC_{50\text{-}90}$=0.5). For the Etest® method, comparison between the 24- and 48-h readings presented an EA of 100 % (Table 1). As caspofungin interpretative breakpoints were available only for the CLSI and Etest® methods, categorical agreement was not calculated when comparing to the EUCAST method. The results of the susceptibilities obtained for the 193 isolates are presented in Table 2. With the CLSI method, 106/193 isolates were found to be intermediate when interpreted with the breakpoint proposed by Pfaller et al. [2], while 191 were susceptible when the slightly modified breakpoint revised by Arendrup et al. [1] was used. Most of the isolates were susceptible with the Etest® whatever the threshold used (192/193, 188/193, and 191/193). Thus, categorical agreement between the two methods was low (36 %) with a large number of major errors (ME=105) (Table 2) when the breakpoint proposed by Pfaller et al. [2] was used for the CLSI method, whereas there was no error with a perfect categorical agreement when the slightly modified breakpoint revised by Arendrup et al. [1] was used for the CLSI method.

Figure 1 represents the repartition of caspofungin MIC 48-h values determined by the Etest®, CLSI, and EUCAST methods for all the isolates, expressed as a percentage. The MIC was ≤0.25 mg/L for 98.4 % and 90.7 % of isolates with the Etest® and CLSI methods, respectively, but only for 26.4 % of isolates with the EUCAST method. Nevertheless, for this latter method, 66.8 % of isolates had an MIC=0.5 mg/L (Table 3), so, finally, 93.2 % had an MIC ≤0.5 mg/L. Only

Table 2 Caspofungin susceptibilities of 193 *Candida glabrata* isolates, categorical agreement, and discrepancies between the Etest® and CLSI methods at 24 h and 48 h according to the threshold used

Threshold used for categorical agreement	Method	Susceptible isolates (*n*) 24 h/48 h	Intermediate isolates (*n*) 24 h/48 h	Resistant isolates (*n*) 24 h/48 h	Categorical agreement (%)[a] 24 h/48 h	Errors		
						VME[b] 24 h/48 h	ME[c] 24 h/48 h	mE[d] 24 h/48 h
Pfaller et al. [2]	CLSI	75/69	104/106	14/18	–	–	–	–
Arendrup et al. [1]	CLSI	192/191	–	1/2	–	–	–	–
Pfaller et al. [2]	Etest	187/188	4/3	2/2	39.9/36.8	13/16	102/105	1/1
Arendrup et al. [1]	Etest	191/191	–	2/2	98.9/100	0/0	1/0	–
S≤2 mg/L, not-S > 2 mg/L	Etest	192/192	0/0	1/1	39.4/36.3	13/17	104/106	0/0

[a] CA calculated between CLSI and Etest methods with 3 different thresholds for Etest

[b] very major error

[c] major error

[d] minor error

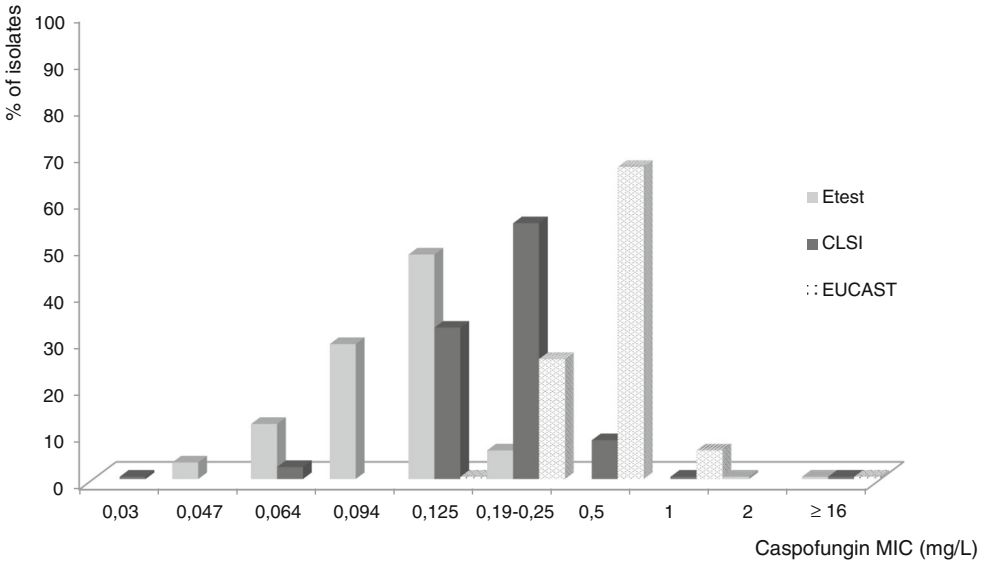

Fig. 1 Repartition of caspofungin MIC 48 h of *Candida glabrata* isolates (as a percentage) according to the method used (Etest®, CLSI, or EUCAST)

two isolates presented high MIC values (≥1 mg/L) with all three methods, and, for one isolate, the MIC value was ≥16 mg/L whatever the method considered (Table 4). Finally, for these two isolates with high MIC values and for the 16 others classified as resistant with the CLSI method and sensitive with the Etest® using breakpoints proposed by Pfaller et al. [2] (corresponding to VME), determination of the presence/absence of *FKS2* or *FKS1* mutations was assessed (see Tables 3 and 4). No *FKS1* mutation was detected in any of the 18 isolates. Regarding the *FKS2* gene, three isolates presented mutations. The two isolates with high MIC values with all three methods showed the presence of previously described mutations: isolate no. 177 (from the urine of a patient hospitalized in the digestive surgery department) presented the mutation of *FKS2*-encoded phenylalanine to tyrosine (F659Y) [10] and isolate no. 120 (from the urine of a patient hospitalized in the intensive care unit) presented an amino acid deletion at phenylalanine 659 (F659del) [11]. Two days after the strain was isolated from urine, patient no. 120 developed candidemia and died in the following few days, despite antifungal therapy. With regards to patient no. 177, he was treated by fluconazole with a favorable outcome. Finally, isolate no. 100 (from the peritoneal fluid of a patient hospitalized in the intensive care unit) with a low MIC value with the Etest® method (0.094 mg/L) but with an MIC of 0.5 mg/L with the CLSI method was found to have a mutation in the *FKS2* gene not yet reported, which consisted in the mutation of phenylalanine to valine (F708V) (Table 4). This patient received no antifungal therapy and had a favorable outcome.

Table 3 Range agreement of caspofungin MIC values between the Etest® and two reference methods according to the following range of MICs: ≤0.25 mg/L, =0.5 mg/L, and ≥1 mg/L

Method		Etest		
	MIC mg/L	≤0.25	= 0.5	≥1
CLSI	≤0.25	175 (90.67 %)	0	0
	= 0.5	16* (8.29 %)	0	0
	≥1	0	0	2* (1 %)
EUCAST	≤0.25	51 (26.4 %)	0	0
	= 0.5	129 (66.83 %)	0	0
	≥1	11 (5.7 %)	0	2* (1 %)

*Isolates with determination of the presence or absence of *FKS1* and *FKS2* mutations

Discussion

Currently, caspofungin is one of the first-line therapies used to treat candidiasis due to *Candida glabrata*. Besides, the Etest® is a recommended method for the routine determination of MIC values [15]. To assess the reliability of our routine practice, the Etest® was compared to the two reference methods of susceptibility determination: the CLSI broth microdilution reference method and the EUCAST method. To do this, the caspofungin susceptibility of 193 *Candida*

Table 4 MIC values (mg/L) obtained with the three different methods for isolates with *FKS2* mutations

	Method			*FKS2* mutation
	Etest	CLSI	EUCAST	
Isolate no. 177	2	1	1	F659Y
Isolate no. 120	32	16	16	F659del
Isolate no. 100	0.094	0.5	0.5	F708V

glabrata isolated from hospitalized patients was evaluated. The Etest® MIC values obtained were in agreement with previous studies [1] and were lower than in both reference methods (see Fig. 1). Also, it is noteworthy that readings at 24 h or 48 h for the CLSI and Etest® methods showed similar results. This suggests that reliable MIC values of caspofungin can be provided at 24 h using the Etest®, even if the 48-h reading remains essential. The MIC results seem to be divided into three categories of values, regardless the method used, but shifted according to the method considered. In regards to the interpretative results obtained with the Etest®, 2/193 (1.04 %) isolates were classified as resistant using breakpoints recommended by the manufacturer, 18/193 (9.33 %) using the breakpoints from Pfaller et al. [2], and 2/193 (1.04 %) using the breakpoint from Arendrup et al. [1]. Interpretative results obtained with the Etest® compared to those with the CLSI method using the breakpoint proposed by Arendrup et al. [1] were totally concordant, whereas when using Pfaller et al.'s breakpoints, 16 VME and 105 ME (corresponding to caspofungin-resistant or -intermediate isolates respectively classified as susceptible by the Etest®) were notified. Nevertheless, all 16 VME corresponded to the CLSI MIC value of 0.5 mg/L, which is the lowest MIC value which classified isolates as resistant. For the 105 ME (corresponding to MIC= 0.25 mg/L with the CLSI method), 83 (79 %) showed Etest® MIC values between 0.094 and 0.19 mg/L. These differences may be due to reading bias, as both the Etest® and CLSI methods required manual reading and can be operator-dependent. Nevertheless, caspofungin powder source, stock solution solvent, etc. [16, 17] could also be involved.

In our work, research into *FKS2* and *FKS1* mutations supports the hypothesis of reading bias for the CLSI method, as only one out of the 16 isolates resistant with the CLSI method and sensitive with the Etest® showed one *FKS2* mutation, which has not yet been described in the literature. Moreover, this mutation is located outside the hotspot region (the end of the hotspot region is around the 660 amino acid region) and, to our knowledge, no echinocandin mutation was ever described in surrounding residues. Supplementary investigations are now necessary to characterize this mutation (F708V) and its eventual implication in resistance. For the two isolates showing high MIC values with the Etest® and reference methods, both presented *FKS* mutations already described in the literature [10, 11].

Finally, in this study, the Etest® was able to detect isolates with *FSK* mutations, known to confer resistance, as well as the CLSI or EUCAST methods. The more relevant breakpoint to interpret the susceptibility of *Candida glabrata* to caspofungin with the Etest® seems to be ≤0.5 mg/mL, as proposed by Arendrup et al. [1].

As caspofungin MIC interlaboratory variability with the CLSI and EUCAST methods was widely notified [16, 17], it was suggested that anidulafungin or micafungin may be more suitable to test echinocandin resistance in epidemiological studies. Nevertheless, for the Etest® method used in routine practice, it can be relevant to test the molecule used to treat the patient. It is an urgent requirement to provide recommendations on the value and interpretation of tests used in routine practice, especially for *Candida glabrata* infections, which are often treated by caspofungin. Another point raised in this study is the end of the reading, as the 24-h end point reading gave similar results to the 48-h reading; this highlighted the interest of the 24-h end point reading, as it allows quicker therapeutic adaptation. Nevertheless, as *Candida glabrata* grows slowly, the 48-h end point reading should still remain recommended.

Acknowledgments The project was supported by investigator-initiated grants to L.L. from Merck. We also acknowledge the technical help of Valérie Fallot, Françoise Grino, Elsa Cazalet, and Myriam Leroyer.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Arendrup MC, Pfaller MA; Danish Fungaemia Study Group (2012) Caspofungin Etest susceptibility testing of Candida species: risk of misclassification of susceptible isolates of C. glabrata and C. krusei when adopting the revised CLSI caspofungin breakpoints. Antimicrob Agents Chemother 56(7):3965–3968
2. Pfaller MA, Messer SA, Moet GJ, Jones RN, Castanheira M (2011) Candida bloodstream infections: comparison of species distribution and resistance to echinocandin and azole antifungal agents in Intensive Care Unit (ICU) and non-ICU settings in the SENTRY Antimicrobial Surveillance Program (2008–2009). Int J Antimicrob Agents 38(1):65–69
3. Pfaller MA, Diekema DJ (2007) Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev 20(1): 133–163
4. Pappas PG, Kauffman CA, Andes D, Benjamin DK Jr, Calandra TF, Edwards JE Jr et al (2009) Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. Clin Infect Dis 48(5):503–535
5. Arendrup MC, Dzajic E, Jensen RH, Johansen HK, Kjaeldgaard P, Knudsen JD et al (2013) Epidemiological changes with potential implication for antifungal prescription recommendations for fungaemia: data from a nationwide fungaemia surveillance programme. Clin Microbiol Infect 19(8):E343–E353
6. Cornely OA, Bassetti M, Calandra T, Garbino J, Kullberg BJ, Lortholary O et al; ESCMID Fungal Infection Study Group (2012) ESCMID* guideline for the diagnosis and management of Candida diseases 2012: non-neutropenic adult patients. Clin Microbiol Infect 18(Suppl 7):19–37
7. Lortholary O, Desnos-Ollivier M, Sitbon K, Fontanet A, Bretagne S, Dromer F et al (2011) Recent exposure to caspofungin or fluconazole influences the epidemiology of candidemia: a prospective multicenter study involving 2,441 patients. Antimicrob Agents Chemother 55(2):532–538
8. Cota JM, Grabinski JL, Talbert RL, Burgess DS, Rogers PD, Edlind TD et al (2008) Increases in SLT2 expression and chitin content are associated with incomplete killing of Candida glabrata by caspofungin. Antimicrob Agents Chemother 52(3): 1144–1146

9. Beyda ND, Lewis RE, Garey KW (2012) Echinocandin resistance in Candida species: mechanisms of reduced susceptibility and therapeutic approaches. Ann Pharmacother 46(7–8):1086–1096
10. Zimbeck AJ, Iqbal N, Ahlquist AM, Farley MM, Harrison LH, Chiller T et al (2010) FKS mutations and elevated echinocandin MIC values among Candida glabrata isolates from U.S. population-based surveillance. Antimicrob Agents Chemother 54(12):5042–5047
11. Garcia-Effron G, Chua DJ, Tomada JR, DiPersio J, Perlin DS, Ghannoum M et al (2010) Novel FKS mutations associated with echinocandin resistance in Candida species. Antimicrob Agents Chemother 54(5):2225–2227
12. Alexander BD, Johnson MD, Pfeiffer CD, Jiménez-Ortigosa C, Catania J, Booker R et al (2013) Increasing echinocandin resistance in Candida glabrata: clinical failure correlates with presence of FKS mutations and elevated minimum inhibitory concentrations. Clin Infect Dis 56:1724–1732
13. Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST) (2008) EUCAST definitive document EDef 7.1: method for the determination of broth dilution MICs of antifungal agents for fermentative yeasts. Clin Microbiol Infect 14(4):398–405
14. Durán-Valle MT, Gago S, Gómez-López A, Cuenca-Estrella M, Jiménez Díez-Canseco L, Gómez-Garcés JL et al (2012) Recurrent episodes of candidemia due to Candida glabrata with a mutation in hot spot 1 of the FKS2 gene developed after prolonged therapy with caspofungin. Antimicrob Agents Chemother 56(6):3417–3419
15. EM Consulte (2004) Prise en charge des candidoses et aspergilloses invasives de l'adulte. Available online at: http://www.em-consulte.com/article/93871/alertePM
16. Espinel-Ingroff A, Arendrup MC, Pfaller MA, Bonfietti LX, Bustamante B, Canton E et al (2013) Interlaboratory variability of Caspofungin MICs for Candida spp. Using CLSI and EUCAST methods: should the clinical laboratory be testing this agent? Antimicrob Agents Chemother 57(12):5836–5842
17. Arendrup MC, Rodriguez-Tudela J-L, Park S, Garcia-Effron G, Delmas G, Cuenca-Estrella M et al (2011) Echinocandin susceptibility testing of Candida spp. Using EUCAST EDef 7.1 and CLSI M27-A3 standard procedures: analysis of the influence of bovine serum albumin supplementation, storage time, and drug lots. Antimicrob Agents Chemother 55(4):1580–1587