Evaluation of the Neo-Sensitabs® diffusion method for determining the antifungal susceptibilities of *Cryptococcus gattii* isolates, using three different agar media

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Summary

The Neo-Sensitabs® diffusion method was evaluated for determining the antifungal susceptibilities of 30 *Cryptococcus gattii* isolates to amphotericin B (AMB), fluconazole (FLC), itraconazole (ITC) and voriconazole (VRC). Three different culture media, Müeller-Hinton (MH), RPMI 1640 (RPMI) and Antibiotic medium 3 (AM3), all supplemented with 2% of glucose and 0.5 µg/ml of methylene blue, were tested. The tests were repeated three times on different days at three incubation times (48, 72 and 96 h). Results were compared with those obtained with the CLSI M27-A2 broth microdilution method. The degree of reproducibility of the diffusion test was 100% for VRC and ITC, 98.3-100% for AMB and 43.3-73.3% for FLC. The best reproducibility was observed at 48 h of incubation and no important differences among media were observed at any of the incubation times assayed. Between Neo-Sensitabs® and the reference method, VRC showed the best agreement and ITC the worst in all conditions tested (100% and 56.7%, respectively). AMB showed a high agreement between the two methods (93.3% to 96.7%) but Neo-Sensitabs® assay failed to detect resistant isolates (discrepancy classified as "very major error") in all times of incubation assayed. Only agreement between both methods for FLC was clearly affected by incubation time and media used, the best results being achieved at 48 h of incubation when MH and RPMI (80.0%, in both media) were used.

Key words Antifungal, Neo-Sensitabs® method, *Cryptococcus gattii*

Evaluación del método de difusión en agar Neo-Sensitabs® para la determinación de la sensibilidad a los antifúngicos de *Cryptococcus gattii*, utilizando tres medios de cultivo diferentes

Resumen

El método de difusión en agar Neo-Sensitabs® fue evaluado en la determinación de la sensibilidad a la anfotericinaB (AMB), fluconazol (FLC), itraconazol (ITC) y voriconazol (VRC) de 30 cepas de *Cryptococcus gattii*. Se utilizaron tres medios de cultivo diferentes: Müeller-Hinton (MH), RPMI 1640 (RPMI) y Antibiotic medium 3 (AM3), todos ellos suplementados con 2% de glucosa y 0,5 µg/ml de azul de metileno. Los ensayos se realizaron tres veces en días diferentes y en tres tiempos de incubación (48, 72 y 96 h). Los resultados fueron comparados con aquellos obtenidos mediante el método de referencia de microdilución CLSI M27-A2. El grado de reproducibilidad del método de difusión fue del 100% para el VRC y el ITC, del 98,3-100% para la AMB y del 43,3-73,3% para el FLC. La mejor reproducibilidad se obtuvo a las 48 h de incubación y no se observaron importantes diferencias entre los distintos medios de cultivo ensayados.

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La mejor concordancia entre el método Neo-Sensitabs® y el de referencia se observó con el VRC y la peor con el ITC (100% y 56,7%, respectivamente), en todas las condiciones estudiadas. La concordancia entre los dos métodos con la AMB fue alta (93,3%-96,7%); sin embargo, el método Neo-Sensitabs® no detectó los aislamientos resistentes (discrepancia clasificada como "very major error") en todos los tiempos de incubación ensayados. Solamente la concordancia entre dichos métodos con el FLC fue dependiente del tiempo de incubación y medio utilizado; los mejores resultados se obtuvieron a las 48 h de incubación, y con los medios MH y RPMI (80%, en ambos medios).

Palabras clave Antifúngicos, Método Neo-Sensitabs®, *Cryptococcus gattii*

Cryptococcus gattii is a basidiomycetous fungus whose natural habitat has been associated with *Eucalyptus camaldulensis* trees and restricted to rural tropical and subtropical areas [12]. This species causes human infections, especially in immunocompetent patients [23], although it also infects immunocompromised patients, especially those who are HIV-positive [1,16]. Amphotericin B (AMB) alone or combined with flucytosine (5FC) remains the standard antifungal therapy for these infections, in spite of the toxicity of both drugs. Other drugs, such as fluconazole (FLC) and itraconazole (ITC) are also used as oral maintenance or consolidation therapy for cryptococcosis [23]. However, some resistance to FLC has arisen in recent years [27]. Voriconazole (VRC) has shown good activity in vitro [32] and excellent efficacy in animal infection by *C. neoformans* [17,22,29], a closely related species, and in a few clinical cases [21,24]. Less data exist on the response to antifungal therapy of *C. gattii* although it seems that the infection caused by this species shows a higher mortality [18,30]. In view of the increasing clinical incidence of *C. gattii* and its poorly known antifungal response, further studies are needed on the activity of the available drugs against this species. A reference method for testing *C. gattii* has so far not been developed. The reference M27-A2 macro- and microdilution reference CLSI methods for antifungal susceptibility testing [9], which have been shown to be very useful for testing *Candida* species and *C. neoformans*, are cumbersome and costly for routine clinical laboratories. Alternative commercial assays such as Sensititre Yeast-One® or Etest® are simpler but expensive. In addition, all these methods need specific equipment and culture media. Therefore, an easy and reproducible screening test, similar to those used for bacteria and able to detect the isolates resistant to antifungal, *in vitro,* would be most advantageous.

The CLSI M44-A standard [8] is a newly established methodology for disk diffusion testing of *Candida* species. This method can be easily implemented in routine clinical microbiology laboratories due its simplicity and low cost. It recommends the use of Mueller-Hinton agar (used in the majority of clinical laboratories for bacteria) supplemented with 0.2% glucose and 0.5 µg/ml methylene blue medium. The drawback, however, is that commercially prepared disks are only available for fluconazole and voriconazole. Neo-Sensitabs® (Rosco Diagnostica A/S, Taastrup, Denmark) is also a simple agar diffusion method for testing yeasts (*Candida* and *Cryptococcus* spp.), which uses tablets to determine the antifungal susceptibility of fungi. This method offers additional advantages, i.e., a large number of antifungals is available and the majority of them can be stored at room temperature. Using this method, different culture media have been tested, such as modified Shadomy agar, Casitone agar, RPMI-1640, and Mueller-Hinton agar alone or supplemented with 2% glucose and 0.5 µg/ml methylene blue [2-6,10,13-15,28,31,33]. Some comparative studies between Neo-Sensitabs® and reference broth dilution methods for testing *Cryptococcus* have been published [2,13,14,31,33]. However, in general, all these studies have focused only on *C. neoformans*. The aim of our study was (i) to evaluate the reproducibility of the Neo-Sensitabs® agar diffusion method for determining the in vitro susceptibilities of *C. gattii* to AMB, FLC, ITC and VRC using three different culture media and three incubation times, and (ii) to compare the results of this diffusion test with those of the reference CLSI broth microdilution method (M27-A2) [9].

Materials and methods

Organisms. Thirty clinical isolates of *C. gattii* were used in this study. The strains were isolated from patients with cryptococcal meningitis in Brazil and maintained in the FIOCRUZ (IPEC/INCQS) Culture Collection, Rio de Janeiro, Brazil. Species were identified using standard methods [11,19]. The isolates were maintained in 20% skimmed milk at –20 ºC until use. *Candida albicans* ATCC 90028, *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality control strains (QC) and included each time that a set of isolates was tested.

Inoculum preparation. Stock fungal inoculum suspensions were prepared in sterile saline from 48 h cultures on Sabouraud dextrose agar at 35 ºC. The suspension was adjusted visually to 0.5 McFarland turbidity standard. Dilutions of these suspensions were subcultured on Sabouraud dextrose agar to determine the number of cfu/ml. The adjusted inoculum was 1×10^6 - 5×10^6 cfu/ml.

Neo-Sensitabs® diffusion method. Tablets containing AMB (10 µg) or ITC (8 µg) or FLC (25 µg) or VRC (1 µg) were supplied by Rosco Diagnostica A/S (Taastrup, Denmark). The following culture media were tested: (i) Müeller-Hinton agar (MH) (Difco, Spain); (ii) RPMI 1640 medium with L-glutamine and without bicarbonate (RPMI) (Gibco BRL, Life Technologies, Izasa, Barcelona, Spain) buffered at pH 7.0 with 0.165 MOPS (Sigma, Spain); and (iii) antibiotic medium 3 (AM3) (Difco, Spain). The two last media were solidified with 1.5% of Bacto agar (Difco, Spain) and all were supplemented with 2% of glucose and 0.5 µg/ml of methylene blue according to CLSI M44-A [8]. The assay was performed according to the manufacturer's instructions [26]. Briefly, the agar plates were inoculated with the suspension using sterile cotton swabs that had been rolled against the side of the tube to remove the excess. The surface of the plates was streaked in three different directions (60º) with the side of the swab. Once the inoculum had been absorbed, antifungal tablets were dispensed

onto the surface of the inoculated agar plates. The plates were inverted and incubated at 35 ºC, and the growth inhibition diameters were measured in millimetres after 48, 72 and 96 h of incubation. For AMB the diameters were measured at points where there was a complete inhibition of growth. For the other of antifungals, measurement was made where there was a prominent inhibition (approximately of 80%) of fungal growth and no colonies of normal size were observed inside the zone. The isolates were classified as susceptible (S) to FLC when the inhibition zone was ≥ 19 mm, resistant (R) when it was ≤ 14 mm and susceptible-dose dependent, (S-DD) when it was between 15 and 18 mm. In the case of VRC the corresponding values were ≥ 17 mm, ≤ 13 mm and 14-16 mm, respectively. The interpretive criteria of R, S and S-DD for both AMB and ITC were, ≥ 15 mm, ≤ 9 mm and 10-14 mm, respectively. To evaluate the reproducibility of the tests they were performed three times on different days.

Reference broth microdilution method. Broth microdilution method was performed according to the CLSI guidelines [9]. Antifungal drugs were obtained as pure powders; AMB from USP, Rockville, MD; ITC from Janssen Pharmaceutica, Beerse, Belgium; and FLC and VRC from Pfizer Inc., Madrid, Spain. The MIC endpoints were read visually after 72 h of incubation at 35 ºC. The AMB MIC was defined as the lowest concentration that produced 100% inhibition of visible fungal growth and 50% for the other antifungals. The breakpoints used for triazoles were those described in the M27-A2. AMB MICs were classified as S, when MICs were ≤ 1 µg/ml, and R, when MICs were ≥ 2 µg/ml.

Data analysis. For reproducibility evaluation, the three values obtained for each isolate-antifungal agent-medium-time of incubation combination were compared. A test was considered reproducible when the three results were in the same category (S, S-DD or R). The correlation between the Neo-Sensitabs® and reference methods was determined as follows: the mode of the three values obtained by Neo-Sensitabs® was scored for each isolate and condition tested; when the three values were different, the median was used. Results were considered in agreement when isolates were classified in the same category (S, S-DD, or R) by both methods. Discrepancies were classified as follows: (i) minor, when one isolate was classified as S or R by one method and as S-DD by the other method; (ii) major, when an isolate was classified as S by the reference method and R by the Neo-Sensitabs® test; and (iii) very major, when an isolate was classified as R by the reference method and S by the Neo-Sensitabs® test [7].

Results

Fungal growth in agar media was more clearly visible after 48 h of incubation when MH and AM3 were used and after 72 h of incubation with RPMI. Trailing growth was observed with most of the strains in almost all media when azoles were tested, but especially with FLC. This problem did not occur with AMB. All strains were considered as S to all antifungals tested according to MICs determined by the reference method, except in the following cases: two strains were considered R to AMB (MIC ≥ 2 µg/ml); seven were considered S-DD to FLC (MIC of 16 to 32 µg/ml); and thirteen were considered S-DD to ITC (MIC of 0.25 to 0.5 µg/ml).

Quality control. Table 1 shows the range of growth inhibition diameters obtained for each QC strain, drug and medium tested and the proposed diameter limits by the Neo-Sensitabs® manufacturer [26]. The diameter zones obtained for all antifungals on MH, with the exception of those obtained for AMB for the three QC strains and for ITC only with *C. parapsilosis* ATCC 22019, were within the expected limits. Diameter zones obtained on RPMI and AM3 media were more variable.

Reproducibility. The percentages of reproducibility obtained at 48, 72 and 96 h of incubation using the three media are shown in table 2. A 100% reproducibility was obtained for ITC and VRC in all the conditions tested. For AMB, reproducibility was 100% both at 48 h of incubation with all media tested and at 72 h of incubation, but only when RPMI and AM3 media were used. For the other conditions tested, the reproducibility for AMB was 98.3%. Reproducibility for FLC was lower and variable depending on the conditions studied. In general, the degree of reproducibility for this drug was higher at 48 h (70.0-73.3%) than both at 72 h (56.7-60.0%) and at 96 h (43.3-56.7%) of incubation. No important differences on reproducibility were observed among the media used.

Table 1. Inhibition zone diameters obtained for each combination of culture medium/antifungal agent and reference strain of *Candida* spp.[a]

Reference Strains	Antifungal drug	Range of zone diameter (mm) with:			Proposed diameter limits[b]
		MH	RPMI	AM3	
C. albicans ATCC 90028	Amphotericin B	22-26	20-23	19-24	18-23
	Fluconazole	28-30	28-32	24-29	28-39
	Itraconazole	24-27	21-26	23-26	21-30
	Voriconazole	31-35	28-34	29-34	31-42
C. parapsilosis ATCC 22019	Amphotericin B	24-31	22-26	24-28	20-26
	Fluconazole	26-33	27-34	24-30	22-33
	Itraconazole	24-28	25-27	24-28	19-26
	Voriconazole	30-35	29-37	31-40	28-37
C. krusei ATCC 6258	Amphotericin B	21-25	19-25	18-23	17-23
	Fluconazole	10-16	10-19	9-16	NA
	Itraconazole	20-22	21-24	21-24	16-22
	Voriconazole	20-23	24-29	26-29	16-25

[a]The tests were performed 12 times on different days.
[b]Diameter limits proposed by Neo-Sensitabs® guidelines in mm on MH media, inoculum McFarland 0.5 undiluted.
NA, not applicable.

Table 2. Reproducibility percentages for 30 isolates of *C. gattii* at three different incubation times and using three different media.

Antifungal drug	Medium	% of reproducible tests[a] at each incubation time		
		48 h	72 h	96 h
Amphotericin B	MH	100	98.3	98.3
	RPMI	100	100	98.3
	AM3	100	100	98.3
Fluconazole	MH	70.0	56.7	53.3
	RPMI	70.0	56.7	56.7
	AM3	73.3	60.0	43.7
Itraconazole	MH	100	100	100
	RPMI	100	100	100
	AM3	100	100	100
Voriconazole	MH	100	100	100
	RPMI	100	100	100
	AM3	100	100	100

[a] The isolates were tested three times on different days. A test was considered reproducible when the three values were in the same category: susceptible (S), susceptible-dose dependent (S-DD) or resistant (R).

Correlation between both methods. Table 3 summarizes the degree of agreement between both methods for each drug, medium and incubation time tested. For AMB, VRC and ITC, similar agreement between both methods was obtained at the different incubation times (93.3-96.7%, 100% and 56.7%, respectively), and no important differences in agreement were observed among media.

Only in the case of FLC, agreement was affected by the incubation time, which was higher at 48 h than both at 72 h and at 96 h of incubation. At 48 h of incubation, better agreement was observed with MH and RPMI (80.0%, in both cases) than with AM3 (73.3%). In contrast, at 72 h and 96 h of incubation, the best agreement was observed with RPMI (66.7% vs. 50.0-63.3%) and with AM3 (53.3% vs. 40-50%), respectively.

Table 3. Agreement between MICs obtained by the CLSI reference method (M27-A2) and 48, 72 and 96 h Neo-Sensitabs® methods for 30 *C. gattii* isolates.

Incubation time	Antifungal drug[a]	Method-medium[b]	No. of isolates by category[c]			No. of isolates with discrepant results[d]			No. (%) of categorical agreement[e]
			S	S-DD	R	Minor	Major	Very major	
48 h	AMB	CLSI	28	–	2				
		MH	30	0	0	0	0	2	28 (93.3)
		RPMI	29	1	0	1	0	1	28 (93.3)
		AM3	29	1	0	1	0	1	28 (93.3)
	FLC	CLSI	23	7	0				
		MH	27	3	0	6	0	0	24 (80.0)
		RPMI	24	5	1	6	0	0	24 (80.0)
		AM3	24	5	1	8	0	0	22 (73.3)
	ITC	CLSI	17	13	0				
		MH	30	0	0	13	0	0	17 (56.7)
		RPMI	30	0	0	13	0	0	17 (56.7)
		AM3	30	0	0	13	0	0	17 (56.7)
	VRC	CLSI	30	0	0				
		MH	30	0	0	0	0	0	30 (100)
		RPMI	30	0	0	0	0	0	30 (100)
		AM3	30	0	0	0	0	0	30 (100)
72 h	AMB	CLSI	28	–	2				
		MH	30	0	0	0	0	2	28 (93.3)
		RPMI	29	1	0	1	0	1	28 (93.3)
		AM3	29	1	0	1	0	1	28 (93.3)
	FLC	CLSI	23	7	0				
		MH	17	7	6	12	3	0	15 (50.0)
		RPMI	20	9	1	10	0	0	20 (66.7)
		AM3	19	6	5	9	2	0	19 (63.3)
	ITC	CLSI	17	13	0				
		MH	30	0	0	13	0	0	17 (56.7)
		RPMI	30	0	0	13	0	0	17 (56.7)
		AM3	30	0	0	13	0	0	17 (56.7)
	VRC	CLSI	30	0	0				
		MH	30	0	0	0	0	0	30 (100)
		RPMI	30	0	0	0	0	0	30 (100)
		AM3	30	0	0	0	0	0	30 (100)
96 h	AMB	CLSI	28	–	2				
		MH	30	0	0	0	0	2	28 (93.3)
		RPMI	29	0	1	0	0	1	29 (96.7)
		AM3	29	0	1	0	0	1	29 (96.7)
	FLC	CLSI	23	7	0				
		MH	14	7	9	12	6	0	12 (40.0)
		RPMI	15	9	6	12	3	0	15 (50.0)
		AM3	16	9	5	13	2	0	16 (53.3)
	ITC	CLSI	17	13	0				
		MH	30	0	0	13	0	0	17 (56.7)
		RPMI	30	0	0	13	0	0	17 (56.7)
		AM3	30	0	0	13	0	0	17 (56.7)
	VRC	CLSI	30	0	0				
		MH	30	0	0	0	0	0	30 (100)
		RPMI	30	0	0	0	0	0	30 (100)
		AM3	30	0	0	0	0	0	30 (100)

[a] AMB amphotericin B, FLC fluconazole, ITC itraconazole and VRC voriconazole.

[b] CLSI, MIC obtained by the CLSI reference method (M27-A2) at 72 h of incubation; MH, RPMI, AM3, inhibition zone diameters determined by the Neo-Sensitabs® method with MH, RPMI and AM3 media, respectively.

[c] Number of isolates classified in the different susceptibility categories according to CLSI MIC and growth inhibition diameter interpretive categories: for fluconazole, MICs ≤ 8 µg/ml or ≥ 19 mm were considered susceptible (S), MICs ≥ 64 µg/ml or ≤ 14 mm were considered resistant (R) and and MICs of 16 to 32 µg/ml or of 15 to 18 mm were considered susceptible-dose dependent (S-DD); for itraconazole, S: ≤ 0.125 µg/ml or ≥ 15 mm, R: ≥ 1 µg/ml o ≤ 9 mm and S-DD: of 0.25 to 0.5 µg/ml or 10 and 14 mm; for voriconazole, S: ≤ 1 µg/ml or ≥ 17 mm, R: ≥ 4 µg/ml or ≤ 13 mm, S-DD: 2 µg/ml or of 14 to 16 mm; for amphotericin B, MICs were classified only in two categories: S, when it was ≤ 1 µg/ml and R ≥ 2 µg/ml, and inhibition zone diameters were classified as follows: S, when its were ≥ 15 mm, R ≤ 9 mm and S-DD between 10 and 14 mm.

[d] Discrepancies were classified as very major errors, when an isolate was classified as R by the reference method (CLSI, M27-A2) and S by the Neo-Sensitabs® test; major errors when an isolate was classified as S by the reference microdilution method and R by the Neo-Sensitabs® test; and minor if an isolate was classified as S or R by one method and as S-DD by the other method.

[e] Categorical agreement reflects the number and percentage of isolates classified in the same category by both Neo-Sensitabs® and CLSI reference method.

When discrepancies for AMB were analyzed (Table 3), two very major errors were observed at each incubation time with MH and one with each of the media RPMI and AM3. Major errors were found only with FLC at 72 h of incubation (three with MH and two with AM3) and at 96 h (six with MH, three with RPMI and two with AM3). No major errors were observed with the other antifungals or conditions tested. Thirteen minor errors were observed for ITC at each incubation time and each media tested. While all these strains were interpreted as S-DD to this antifungal by the broth reference microdilution method, they were interpreted as S by the Neo-Sensitabs® method. In the case of FLC, more variability was observed in the analysis of their minor errors. MH showed six minor errors at 48 h of incubation and twelve, at each 72 and 96 h of incubation. The minor errors observed with the other two media tested corresponding to these three incubation times were six, ten and twelve with RPMI, and eight, nine and thirteen, with AM3, respectively.

Discussion

The susceptibility of *C. gattii* to the common drugs used in the treatment of cryptococcosis has been determined under different testing conditions. 5FC was not included in this study as all strains previously tested by Neo-Sensitabs® were highly resistant to this drug (growth inhibition diameters ≤ 9 mm) (unpublished data).

Although the reproducibility of results is one of the most striking problems in standardizing antifungal susceptibility tests, in most of the studies published on the evaluation of the Neo-Sensitabs® method this has not been a significant issue [10,15,28,31,33]. In other studies, reproducibility analysis was only performed with a small set the isolates [2,13,14] and, in general, our results agree. In our study, the best reproducibility was obtained at 48 h of incubation contrasting with the 72 h reported by Espinel-Ingroff et al. [14], and the worst reproducibility was observed for FLC, probably due to the presence of the mentioned trailing effect. This phenomenon impedes a clear definition of the inhibition zone edges and can cause variability of results. Therefore, interpretation of the precise meaning of "growth of partially inhibited" recommended by the manufacturer of Neo-Sensitabs® [26], or "a prominent reduction of fungal growth" recommended by CLSI M44-A [8], or "growth inhibition of approximately 80%" (mainly applied for the majority of authors) in the reading of inhibition zones, remains problematic. Automatic plate reader systems such as BIOMIC [20,25] may reduce the effect of subjectivity of reading, but they are not available in most clinical laboratories.

Although in our study excellent correlation between both methods was achieved for AMB, which agrees in general with others authors [10,14,31], the Neo-Sensitabs® method failed to detect resistant isolates and very major errors were detected with this drug. Such errors were only found with this antifungal. Recently, Espinel-Ingroff et al. [14] testing *C. neoformans* and *Candida* spp. used a different criterion for categorizing the results (diameters of ≥ 15 mm: S and ≤ 13 mm: R) and found a similar agreement between both methods for AMB (98.2%). The use of that criterion in our case would have improved the reproducibility of the Neo-Sensitabs® results (100% in all conditions of testing) but not the agreement between the methods. However, no *Cryptococcus* isolates classified as resistant by broth reference method were tested in any of the mentioned studies [10,14,31].

Concerning the azoles, only one previous report has evaluated the Neo-Sensitabs® method for testing VRC in yeasts and a high agreement between the methods has also been found (agreement > 95.5% with only minor errors) [14]. It is difficult to compare our results for ITC with those published previously due to diversity of the methods used. Two previous studies have reported percentages of agreement from 66% to 76.4%, with very major discrepancies (1%-1.1%) between the methods [10,31]. In contrast, the above mentioned study of Espinel-Ingroff et al. [14], which also used different criteria to classify the isolates as "susceptible" or "resistant" to ITC (S = diameters of ≥ 23 mm, S-DD = 14 to 22 mm, and R ≤ 13 mm), reported higher agreement (87.3% with MH and 92.7% with RPMI 2% dextrose media) with only minor errors (7.3% and 12.7%, respectively). However, the application of these criteria in our work did not improve the agreement for this drug. FLC has been the triazole most commonly evaluated with the Neo-Sensitabs® method [2,10,14,15,28,31,33] but generally, in those studies, tablets with 15 µg of FLC, media without glucose and methylene-blue and different criteria for classifying the isolates as "susceptible" or "resistant" were used. Although a lower percentage of agreement between both methods were observed (< 80%), most of these studies reported the ability of the Neo-Sensitabs® method for detecting *Candida* strains susceptible to this drug with high positive predictive and specificity values. However, resistant isolates detected by this method should be further investigated by a reference method in order to confirm the resistance [15,28,33]. Recently, other studies similar to ours, described agreement between Neo-Sensitabs® and reference methods to testing FLC from 83.1 to 96.4% [2,10,14] with no important differences between the results obtained with MH and RPMI 2% dextrose media with or without methylene blue supplementation [2,14]. However, in some of these studies, discrepancies classified as very major errors were reported [2,10].

In conclusion, the commercial Neo-Sensitabs® diffusion method is a promising assay for in vitro antifungal susceptibility testing of yeasts including *C. gattii*, but further studies using isolates with high in vivo antifungal resistance are needed to confirm the usefulness of this method in a routine laboratory.

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