Multicenter evaluation of Neo-Sensitabs, a standardized diffusion method for yeast susceptibility testing

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The agar diffusion method Neo-Sensitabs for sensitivity testing, was evaluated with 33 reference strains by fourteen laboratories. Tablets with 5-fluorocytosine, amphotericin B, nystatin, fluconazole, itraconazole, ketoconazole and tioconazole were used on Shadomy modified medium. These tests classify each strain as susceptible, intermediate or resistant to all tested antifungals by measuring the inhibition zone diameters. Intra and interlaboratory reproducibility was studied. Neo-Sensitabs sensitivity for fungi was easy to perform and reliable method with a reproducibility of 97.1% and superior to other commercialized methods, being specially interesting for antifungal susceptibility *in vitro* testing of triazole derivatives fluconazole and itraconazole.

Key words Antifungal susceptibility, Neo-Sensitabs, Collaborative study

Evaluación multicéntrica de Neo-Sensitabs, método de difusión estandarizado para pruebas de sensibilidad en levaduras

Se evaluó el método de difusión en agar con tabletas Neo-Sensitabs con 33 cepas de referencia en14 laboratorios. Se emplearon tabletas con 5-fluorocitosina, anfotericina B, nistatina, fluconazol, itraconazol, ketoconazol y tioconazol en un medio de Shadomy modificado. Las cepas son clasificadas como sensibles, intermedias o resistentes a los antifúngicos evaluados en base al diámetro del halo de inhibición del crecimiento. Se estudió la reproducibilidad dentro de cada laboratorio y entre los diferentes laboratorios. La sensibilidad antifúngica fue sencilla de determinar y el método se mostró eficaz con una reproducibilidad del 97,1% y superior a otros métodos comercializados, siendo de gran interés para evaluar *in vitro* la actividad antifúngica de los derivados triazólicos fluconazol e itraconazol.

Sensibilidad antifúngica, Neo-Sensitabs, Estudio colaborativo

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Incidence of superficial and invasive opportunistic mycoses is increasing, caused by a wide range of pathogenic fungi, specially in relation with AIDS and other immunocompromised patients [1,2]. The appearance of new antifungal drugs including triazole derivatives fluconazole and itraconazole, during the last years [3,4], offers new perspectives for the treatment of these infections.

In vitro antifungal susceptibility testing is justified and indicated under certain clinical conditions, specially for pathogenic yeasts [5] isolated from immunocompromised or neutropenic patients [6,7] in clinical failures of antifungal therapy and in recurrent mucocutaneous mycoses [2]. The evaluation of antifungal activity *in vitro* against these isolates should be predictive of the clinical response, allowing the clinician to choose the most suitable treatment. Nevertheless, antifungal susceptibility tests are not standardized, and influenced by numerous factors such as yeasts species, antifungal agents tested and technical factors. We have to consider adequately inoculum size, conditions of incubation, solvents, reading of endpoints and interpretative criteria to achieve a correct characterization of susceptibility [7-11]. These factor have influence in susceptibility results and *in vitro* activity of antifungal agents, that should be interpreted with care.

A useful standardized method must be updated when new generations of antifungal drugs will appear. Macro or micro-dilution methods proposed by the NCCLS [8,9] could be difficult to introduce in the routine of the clinical laboratories without a special training, in spite of the high degree of standardization achieved. Therefore commercial methods became an effective tool in the evaluation of susceptibility [7]. Neo-Sensitabs was designed as an antibacterial susceptibility method, and later modified to test the susceptibility of yeasts to antifungal agents [12]. The aim of this collaborative study has been to evaluate the reproducibility of this easy to perform and rapid agar diffusion assay, involving fourteen laboratories and using reference fungal strains used in previous collaborative studies [13,14]. The antifungals tested were amphotericin B, 5-fluorocytosine, as reference substances, and nystatin and tioconazole, because of their excelent *in vitro* activity against yeasts and the azoles fluconazole, itraconazole and ketoconazole.

MATERIALS AND METHODS

Strains. The collection was selected according to its importance in human pathology and their pattern of susceptibility to antifungal agents. We included 23 strains from the Universidad del País Vasco yeast collection (UPV) and seven strains from the British National Collection of Pathogenic Fungi (NCPF). Three quality control strains (*Candida albicans* ATCC 64548, *Candida albicans* ATCC 64550 and *Candida glabrata* 2238NL) for Neo-Sensitabs sensitivity testing of fungi, provided by Rosco® (Taastrup, Denmark), were also included. The species represented were: *Candida albicans* (nine strains), *Candida famata* (one strain), *Candida glabrata* (three strains), *Candida guilliermondii* (two strains), *Candida kefyr* (one strain), *Candida parapsilosis* (two strains), *Candida tropicalis* (four strains), *Candida rugosa* (one strain), *Candida viswanathii* (one strain), *Cryptococcus laurentii* (one strain), *Cryptococcus neoformans* (two strains), *Hansenula anomala* (one strain) and *Trichosporon cutaneum* (two strains). These strains were codified and distributed to the different laboratories. The origin of the strains is summarized in Table 1, where the reference minimal inhibitory concentration (CMI) is included for amphotericin B, 5-fluorocytosine, nystatin and ketoconazole, previously determined at the Universidad del País Vasco [14] as described by Espinel-Ingroff *et al.*[15]. Quality control limits of diameter inhibition zones on Shadomy modified agar are included in Table 2.

Neo-Sensitabs susceptibility testing. Neo-Sensitabs® sensitivity testing is a standardized agar diffusion method developed by Rosco® (Taastrup, Denmark), which includes nineteen different antifungal agents in tablets of 9 mm of diameter, allowing to choose the adequate antifungal agents for every test. In this collaborative

Table 1. Reference strains used and MCI´s (µg/ml), using a microdilution method in RPMI 1640 buffered with MOPS (Espinel-Ingroff *et al.* 1991). Data from Quindós *et al.* 1994. AMB= amphotericin B, NYS= nystatin, 5FC= 5-flucytosine, KTZ= ketoconazole.

N.	*Identification*	Clinical sample	AMB	NYS	5FC	KTZ
1	*Cryptococcus neoformans*	CSF	1	2	8	0.25
2	*Trichosporon cutaneum*	Skin	2	4	64	2
3	*Rhodotorula rubra*	Rectum	2	1	0.25	0.25
4	*Candida rugosa*	Rectum	2	4	1	0.25
5	*Candida albicans*	Blood	2	2	0.25	32
6	*Candida albicans*	Vagina	2	4	16	64
7	*Cryptococcus neoformans*	NCPF 3170	1	1	16	0.25
8	*Trichosporon cutaneum*	Skin	2	4	32	2
9	*Candida albicans*	NCPF 3153	2	4	0.25	64
10	*Candida albicans*	Oral cavity	2	4	0.25	32
11	*Candida glabrata*	Oral cavity	2	2	0.25	0.25
12	*Candida tropicalis*	Oral cavity	1	2	0.5	64
13	*Candida krusei*	NCPF 3100	2	4	64	1
14	*Candida viswanathii*	NCPF 3151	1	2	0.5	16
15	*Candida kefyr*	NCPF 3106	4	2	0.25	16
16	*Candida kefyr*	Oral cavity	4	2	4	64
17	*Candida albicans*	Kidney	2	2	0.5	64
18	*Candida albicans*	Blood	2	2	0.25	32
19	*Candida glabrata*	Vagina	4	2	0.25	1
20	*Candida krusei*	Blood	4	4	64	4
21	*Candida guilliermondii*	Blood	4	2	0.25	0.25
22	*Candida parapsilosis*	Liver	4	4	0.5	0.25
23	*Hansenula anomala*	Blood	2	4	0.25	1
24	*Cryptococcus laurentii*	NCPF 3141	4	4	0.25	0.25
25	*Candida parapsilosis*	Urine	4	8	1	0.25
26	*Candida tropicalis*	Urine	4	4	0.25	16
27	*Candida tropicalis*	Urine	2	4	0.5	16
28	*Candida tropicalis*	Urine	2	4	0.25	16
29	*Candida famata*	Vagina	2	8	0.25	1
30	*Candida guilliermondii*	Blood	*	*	*	*
31	*Candida albicans*	ATCC 64548	*	*	*	*
32	*Candida albicans*	ATCC64550	*	*	*	*
33	*Candida glabrata*	2238 NL	*	*	*	*

* No evaluated by this method in previoulsy report (14).

Table 2. Control limits on modified Shadomy agar (in mm) for quality control strains. Data provided by one laboratory. AMB= amphotericin B, 5FC= 5-flucytosine, FZN= fluconazole, ITR= itraconazole, KTZ= ketoconazole.

Antifungal agent	*Candida albicans* ATCC 64548	*Candida albicans* ATCC 64550	*Candida glabrata* 2238 NL
AMB	18-23	19-24	9-13
5FC	34-40	11-17	24-30
FZN	36-42	28-34	47-53
ITR	25-31	0	22-30
KTZ	36-42	23-29	28-34

study, we included 5-fluorocytosine (1 µg) amphotericin B (10 µg), fluconazole (15 µg), itraconazole (10 µg), ketoconazole (15 µg), nystatin (50 µg), and tioconazole (10 µg) tablets. Inocula contained $5x10^5$ cells/ml and were prepared from an overnight subculture on Sabouraud glucose agar (Difco, USA), getting suspensions corresponding to McFarland 0.5 standard and then diluted (1+1) with sterile saline solutions. For *Candida krusei* isolates inocula were equivalent to McFarland 0.5 standard, diluted 1:10 in saline solutions. Volumes of 2 ml of each inoculum suspensions were poured onto the agar surface flooding the excess of liquid and removing it immediately with a Pasteur pipette. Opened plates were dried at 35∞C for 15 min and tablets were placed on the agar surface. Incubation of plates was made at 35∞C (30∞C was used for *Cryptococcus* spp).

Shadomy modified medium was used (Yeast Nitrogen Base, asparagine and glucose) with phosphate buffer to pH 7 containing cloramphenicol to avoid bacterial contamination. Bottles of 50 ml and tablets corresponding to the same batches were supplied by the manufacturer. Bottles were heated at 100∞C during 15 min, and then cooled to 60∞C before pouring the medium in Petri dishes (12 cm x 12 cm). A pre-diffusion drying was made at 37∞C during 20 minutes.

After an incubation period of 20-24 h, the inhibition diameter zones were read. A reincubation of 24 h was made in case the growth was not visible. For 5-fluorocytosine and polyenes the clear visible inhibition zone (with no colonies inside it) was measured. For the azole derivatives we measured the zone up to colonies of normal size.

This test allows the categorization of the strains into susceptible, intermediate or resistant, according to the zone diameter interpretative standards. For 5-fluorocytosine, fluconazole, ketoconazole and tioconazole susceptible strains were those with diameter zones of more or equal to 20 mm; intermediate, between 12 mm and 19 mm; and resistant, with less than 12 mm. For amphotericin B, nystatin and itraconazole, susceptible strains were those with diameter zones more or equal to 15 mm; intermediate, between 10 mm and 14 mm; and resistant those with no zone at all. A more strict interpretative criteria are recommended with isolates from serious systemic mycoses, to detect strains with reduced sensitivity.

Data analysis. For reproducibility evaluation the tests were performed three times on three different days in each laboratories. A total amount of 9,702 yeast-antifungal combinations were obtained. Data including inhibition zone diameters (9,400) and results of susceptibility were processed with SPSS PC+ for IBM and SAS. We did not introduce in the statistic study those yeast-antifungal combinations not tested by triplicate. Differences between results for each strain were also studied. Major discrepancies were defined when results for the same strain changed from resistant to sensitive (or viceversa), and minor discrepancies when variations were detected between intermediate and sensitive or between intermediate and resistant for a given strain.

RESULTS

The average reproducibility was 97.1% for 9,702 antifungal-yeast combinations, ranging between 93.3% and 100% obtained in two laboratories (Table 3). Only 9400 (96.9%) data were processed, due to mistakes in manipulation of plates or tablets, deviation from the protocol or incorrect interpretation of the inhibition zones. Overall reproducibility among antifungal agents was: 97.9% for amphotericin B, 97.5% for 5-fluorocytosine, 97.1% for fluconazole, 96.1% for itraconazole, 98.1% for nystatin, 97.5% for ketoconazole and 97.9% for tioconazole. Analysis of variance of inhibition zone diameter showed the presence of significant ($p<0.05$) statistical difference among laboratories for all antifungal agents except for itraconazole. But when interpretative criteria were used, no statistical difference was found between the susceptibility results obtained with fluconazole, itraconazole, nystatin, ketoconazole and tioconazole in the 14 laboratories. Reproducibility intra and interlaboratory was conditioned by the strain tested (Table 4). For azole derivatives, a significant less concordance was obtained with *Candida krusei* strains (No. 13 and 20) that showed the highest number of major discordances. Intralaboratory reproducibility of susceptibility, considering concordance of triplicate results, ranged between 420 cases (amphotericin B, nystatin and tioconazole) and 396 cases for itraconazole (Table 5).

Table 3. Reproducibility (%) of Neo-Sensitabs susceptibility testing for 9,702 yeast-antifungal combinations. AMB= amphotericin B, 5FC= 5-fluorocytosine, FZN= fluconazole, ITR= itraconazole, NYS= nystatin, KTZ= ketoconazole, TZN= tioconazole.

Laboratory	AMB	5FC	FZN	ITR	NYS	KTZ	TZN	TOTAL
1	93.9	91.9	95.9	93.9	95.9	89.9	91.9	93.32
2	97.0	99.0	97.0	98.0	98.0	100	94.9	97.7
3	97.0	98.0	98.0	97.0	98.0	99.0	100	98.14
4	96.0	91.9	94.9	95.9	99.0	97.0	95.9	95.8
5	99.0	96.9	93.9	89.6	99.0	94.9	95.9	95.6
6	95.8	95.8	93.7	91.9	97.9	97.9	99.0	96.0
7	96.0	93.9	95.8	94.9	93.9	95.9	99.0	95.62
8	98.9	96.7	94.4	96.5	96.7	98.9	98.9	97.28
9	98.9	100	97.8	95.8	100	98.9	98.9	98.61
10	100	99.0	99.0	96.0	98.0	97.0	99.0	98.28
11	98.9	98.9	100	97.8	97.8	98.9	100	98.9
12	100	100	100	100	100	100	100	100
13	100	100	98.9	98.9	98.9	98.9	97.9	99.07
14	100	100	100	100	100	100	100	100
TOTAL	97.9	97.5	97.1	96.1	98.1	97.5	97.9	97.1

Results with control strains showed a better agreement between laboratories with amphotericin B (average of 87.4%), fluconazole (average of 62.7%) and itraconazole (average of 65.8%) (Table 6). Percentage indicated the number of assays inside the reference limits provided by the manufacturer.

DISCUSSION

We present a contribution to the standardization of *in vitro* susceptibility of yeasts using a commercialized test. The agar diffusion methods have previously been reported by an adequate correlation with microdilution or agar dilution methods [16-18]. Neo-Sensitabs susceptibility testing for fungi, had an excellent reproducibility ranging between 98.1% with nystatin and 96.1% with itraconazole and should be considered a useful method. It seems to be dependent on the inoculum size and time of incubation. This probably was the source of variation introduced by the strains tested. Strains with a wide diversity of requirements, like time of incubation or temperatu-

Table 4. Reprodiucibility of Neo-Sensitabs by strains. C= concordance, MD= major discrepances and md= minor discrepances.

	AMB			5FC			FZN			ITR			NYS			KTZ			TZN		
Strain	C	MD	md	C	MD	md	C	MD	md	C	MD	md	C	MD	md	C	MD	md	C	MD	md
1	14	-	-	10	-	2	13	1	-	14	-	-	14	-	-	14	-	-	14	-	-
2	13	1	-	12	2	-	14	-	-	13	-	1	14	-	-	13	-	1	14	-	-
3	13	-	-	10	1	2	9	3	1	7	4	2	13	-	-	12	1	-	7	1	5
4	14	-	-	14	-	-	12	2	-	13	1	-	13	-	1	14	-	-	14	-	-
5	14	-	-	13	1	-	14	-	-	12	1	1	12	-	2	13	-	1	14	-	-
6	12	-	-	13	1	-	14	-	-	14	-	-	14	-	-	14	-	-	14	-	-
7	14	-	-	12	1	1	12	1	1	14	-	-	14	-	-	14	-	-	14	-	-
8	9	1	2	13	1	-	11	-	2	12	-	1	7	-	6	9	-	4	12	1	-
9	12	-	-	14	-	-	14	-	-	14	-	-	11	-	3	14	-	-	14	-	-
10	13	-	3	14	-	-	14	-	-	13	-	1	13	-	1	13	-	1	14	-	-
11	14	-	2	12	-	2	14	-	-	14	-	-	14	-	-	13	-	1	14	-	-
12	14	-	1	14	-	-	12	1	1	10	2	2	14	-	-	13	-	1	12	-	12
13	13	-	-	13	-	-	8	-	5	8	-	5	13	-	-	6	-	7	10	-	3
14	14	-	-	12	1	1	13	-	1	13	1	-	13	-	1	12	1	1	13	-	1
15	14	-	-	14	-	-	14	-	-	14	-	-	14	-	-	14	-	-	14	-	-
16	14	-	-	10	1	3	14	-	-	14	-	-	14	-	-	14	-	-	14	-	-
17	14	-	-	14	-	-	14	-	-	14	-	-	14	-	-	14	-	-	14	-	-
18	14	-	-	14	-	-	14	-	-	14	-	-	14	-	-	14	-	-	14	-	-
19	14	-	-	13	1	-	10	-	4	10	3	-	14	-	-	12	-	2	14	-	-
20	13	-	1	13	1	-	10	1	3	6	3	5	14	-	-	5	1	8	9	-	5
21	14	-	-	14	-	-	12	1	1	12	2	-	14	-	-	14	-	-	14	-	-
22	12	-	1	14	-	-	14	-	-	13	1	-	14	-	-	14	-	-	14	-	-
23	11	1	-	11	-	1	12	-	-	12	1	-	11	-	1	12	-	-	11	1	-
24	12	-	-	12	-	-	12	-	-	11	-	1	11	1	-	12	-	-	12	-	-
25	12	-	2	14	-	-	14	-	-	14	-	-	14	-	-	14	-	-	14	-	-
26	12	1	1	12	2	-	14	-	-	14	-	-	14	-	-	14	-	-	14	-	-
27	11	1	2	12	1	1	14	-	-	14	-	-	12	1	1	14	-	-	14	-	-
28	14	-	-	13	1	-	14	-	-	12	1	1	13	-	-	14	-	-	13	-	1
29	12	-	2	14	-	-	14	-	-	13	1	-	13	-	-	14	-	-	14	-	-
30	14	-	-	14	-	-	14	-	-	14	-	-	14	-	-	14	-	-	14	-	-
31	8	2	1	9	1	-	8	1	2	7	-	4	6	2	3	11	-	-	10	-	1
32	10	-	2	8	2	2	7	5	-	11	-	1	10	2	2	10	-	2	8	-	4
33	12	-	-	12	-	-	10	1	-	11	-	1	12	-	-	12	-	-	11	-	1

Table 5. Intralaboratory reproducibility of susceptibility by Neo-Sensitabs. Numbers correspond to cases. C= concordance, MD= major discrepances, md= minor discrepances.

	AMB			5FC			FZN			ITR			NYS			KTZ			TZN		
Laboratory	C	MD	md	C	MD	md	C	MD	md	C	MD	md	C	MD	md	C	MD	md	C	MD	md
1	27	4	2	27	5	1	29	2	2	29	3	1	29	1	3	25	2	6	25	2	6
2	30	-	3	32	1	1	32	1	-	31	-	2	31	-	2	33	-	-	30	1	2
3	30	-	3	31	-	-	31	-	2	30	-	3	31	-	2	32	-	1	33	-	-
4	29	-	4	25	5	2	28	-	5	29	-	4	32	-	1	30	-	3	29	-	4
5	32	-	1	29	-	3	27	-	6	22	7	3	32	-	1	28	-	4	29	-	3
6	28	3	2	28	4	3	26	1	5	25	4	4	30	2	-	30	-	2	31	-	1
7	29	2	2	27	6	-	28	2	2	28	3	2	27	2	4	29	-	4	31	-	1
8	29	-	-	27	-	-	25	3	2	26	1	2	27	-	3	29	-	1	29	-	1
9	31	-	2	31	-	3	29	2	-	28	3	1	31	-	-	30	-	1	30	-	1
10	33	-	-	32	1	-	32	-	1	28	1	1	31	1	1	31	-	2	32	-	1
11	29	-	4	29	-	-	30	-	-	28	-	2	28	-	2	29	-	1	30	-	-
12	28	-	5	28	-	1	28	-	-	28	-	-	28	-	-	28	-	-	28	-	-
13	32	-	1	32	-	-	31	-	1	31	1	-	31	-	1	30	-	2	30	-	2
14	33	-	-	33	-	-	33	-	-	33	-	-	32	-	-	33	-	-	33	-	-
Total	420	9	29	411	22	14	409	11	46	396	23	25	420	6	20	417	2	27	420	3	22

Table 6. Results with quality control strains. Percentage indicates the number of assays inside the reference limits provided by the manufacturer. AMB= amphotericin B, 5FC= 5-fluorocytosine, FZN= fluconazole, ITR= itraconazole, KTZ= ketoconazole.

	Candida albicans ATCC 64548		*Candida albicans* ATCC 64550		*Candida glabrata* 2238 NL	
Antifungal agent	Inside	Out	Inside	Out	Inside	Out
AMB	78.9	2.9	86.8	13.2	97.1	2.9
5FC	57.9	42.1	13.2	86.8	28.6	71.4
FZN	62.2	37.8	84.2	15.8	40	60
ITR	71.2	28.9	100	-	22.9	77.1
KTZ	50	50	31.6	68.4	45.7	54.3

re could influence the diameter size of the inhibition zones [7]. Differences between laboratories among inhibition zones diameter was probably dependent of the quantification system used for the inoculum, because visual turbidity was employed. A bad interpretation or different inoculum sizes could be related with problems appeared with 5-fluorocytosine, when colonies were found inside the inhibition zone. All these variables can affect other *in vitro* susceptibility methods and have been described previously [19,20].

In conclussion, this multicenter evaluation demonstrates the excellent reproducibility of the Neo-Sensitabs tablets-based susceptibility testing method for fungi. In addition, its low prize and the possibility of including the last antifungal derivatives, makes of this easy-to-perform method a good alternative for the antifungal susceptibility testing of yeasts. The Neo-Sensitabs showed a higher reproducibility in comparison with other methods such as ATB-fungus (95.4%) [14] and Candifast [13] in studies performed with the same collection of refe-

rence strains, but similar to those reported by Guinet *et al.* for ATB-fungus [21], being the main advantage the incorporation of fluconazole and itraconazole in the Neo-Sensitabs system.

Nevertheless, further studies are required to evaluate its correlation and concordance with micro and macrodilution methods proposed by NCCLS, and automatization of inocula. If an adequate correlation could be found with them, Neo-Sensitabs method could be a predictive system to know the sensitivity of yeast to antifungal agents, specially indicated for those laboratories with little experience in the field of *in vitro* sensitivity testing of fungi.

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