

Article

A Comparative Prospective Study in Evaluating *Candida* spp. In Vitro Susceptibility through Micronaut-AM and Sensititre Yeast-One

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Abstract: Background. Among invasive fungal infection pathogens, *Candida* spp. represent the most common aetiological agents. The increasing rate of severe infections and the emergence of antimicrobial resistance highlight the importance of in vitro susceptibility testing. The EUCAST and the CLSI have established reference microdilutions that are reliable but difficult to apply in a laboratory routine. Commercial microdilutions could represent a valuable alternative within a diagnostic workflow. Methods. A number of 50 *Candida* spp. collected from positive blood samples simultaneously underwent the Sensititre Yeast-One microdilution as a standard susceptibility test and the Micronaut-AM as an experimental method. A comparison between the two techniques was produced, evaluating the effectiveness of the Micronaut-AM compared to the extensively consolidated Sensititre Yeast-One. Results. The two techniques revealed optimal agreement rates, confirming the reliability of the commercial microdilution kits within the diagnostic workflows. The results showed remarkable concordance for both susceptible and resistant isolates, highlighting slight variations in the different identified *Candida* species. Conclusions. Future studies about antifungal susceptibility testing should be encouraged, including molecular confirmation of possible resistance phenotypes and extended isolate numbers for the different *Candida* species. Moreover, it would be interesting to plan clinical trials after the execution of the examined commercial microdilution methods.

Keywords: antifungal susceptibility; broth microdilution; *Candida* spp.; Micronaut-AM



Citation: Trovato, L.; Calvo, M.; Scalia, G.; Oliveri, S. A Comparative Prospective Study in Evaluating *Candida* spp. In Vitro Susceptibility through Micronaut-AM and Sensititre Yeast-One. *Microbiol. Res.* **2023**, *14*, 1077–1088. <https://doi.org/10.3390/microbiolres14030072>

Academic Editor: Hector M. Mora-Montes

Received: 28 June 2023

Revised: 2 August 2023

Accepted: 5 August 2023

Published: 7 August 2023



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1. Introduction

Opportunistic fungal pathogens recently reached concerning infection rates, causing an extended disease spectrum. *Candida* species represent the most common systemic infection aetiological agents, and antimicrobial resistance is currently an increasing problem among this fungal genus [1,2]. According to the most recent Centers for Disease Control and Prevention (CDC) epidemiological evaluation, *Candida* spp. may express azole resistance, particularly in *Candida parapsilosis*, *Candida glabrata*, and *Candida auris* [2]. Furthermore, recent literature has documented echinocandin resistance in *Candida glabrata*, *Candida albicans*, and *Candida parapsilosis* [3–5]. All the consulted data highlighted the presence of antimicrobial resistance in bloodstream isolates, complicating patients' clinical and therapeutic management [5–7]. Patients affected by fungal pathogens suffer from significant risk factors such as immunosuppression, broad-spectrum long-term antibiotic or steroid treatments, and medical device implantation. These assumptions suggest possible disadvantageous outcomes, especially in the case of resistant isolate detection. Thus, it is essential to correctly predict the antimicrobial treatment response using reproducible testing [2,8]. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) have established reference

methods to test antifungal susceptibility through broth microdilution [9,10]. However, these standard methods require long intervals, lots of manual work, and dedicated expertise. Therefore, commercial kits such as The Sensititre Yeast-One (Thermo Fisher Scientific, Waltham, MA, USA) and the MICRONAUT-AM (MERLIN Diagnostika GmbH, Bornheim, Germany) can provide a reliable microdilution minimum inhibitory concentration (MIC) value [1,11].

The present study compared Micronaut-AM and Sensititre Yeast-One in defining reliable MIC values in *Candida* species. The fundamental study purpose was the planning of a prospective study, which allowed to avoid reversions in antifungal drug in vitro susceptibility already described within literary data [12]. Simultaneously, the conventional diagnostic workflow defined the susceptibility profiles for all the tested strains.

2. Materials and Methods

2.1. Sample Size and Experimental Design

This 8-months prospective study (October 2022–April 2023) was conducted in the University Hospital Policlinico of Catania. The study included a number of 50 *Candida* strains isolated from positive peripheral blood samples from patients recovered within Internal Medicine, Haematology, and Intensive care units. The collected blood samples were incubated in Bactec FX Top Unit (Beckton Dickinson, Franklin Lake, NJ, USA), whose positive flag was followed by an extemporaneous Gram stain. The detection of yeasts was continued by a culture on Sabouraud Dextrose agar plates with 2% glucose (Vakutest Kima, Arzergrande, Italy), incubated for at least 24–72 h at a temperature of 37 °C. The MALDI Biotyper[®] Sirius System (Bruker, Billerica, MA, USA) identified yeast-grown colonies. All the identified strains underwent antifungal susceptibility testing by Sensititre Yeast-One as the standard laboratory routine procedure (Thermo Fisher Scientific, Waltham, MA, USA) and by MICRONAUT-AM (MERLIN Diagnostika GmbH, Bornheim, Germany) as the experimental evaluation. The interpretation of the obtained MIC values was performed with caution due to the possibility of trailing effects. At the same time, each procedure used *Candida parapsilosis* ATCC22019 and *Candida krusei* ATCC6258 as quality control. All the possible resistance or non-wild-type (non-WT) MIC values were confirmed through EUCAST (for Micronaut-AM) or CLSI (for Sensititre Yeast-One) standard microdilution methods [8,9]. Particularly, standard reference broth microdilution verified resistance MIC values due to significant interlaboratory variability using routine testing [13–15]. The study was a non-interventional protocol, and the patients did not undergo supplementary sample collections or invasive procedures. All the investigations regarded the same samples, which underwent the standard diagnostic workflow, intervening only on grown microorganism isolates and never on human beings.

2.2. Sensititre Yeast-One

The Sensititre Yeast-One is a commercial microdilution method including anidulafungin (0.015–8 mg/L), micafungin (0.008–8 mg/L), caspofungin (0.008–8 mg/L), fluconazole (0.12–256 mg/L), posaconazole (0.008–8 mg/L), voriconazole (0.008–8 mg/L), itraconazole (0.015–16 mg/L), amphotericin B (0.12–8 mg/L), and 5-fluorocytosine (0.06–64 mg/L). All the antifungal drug dilutions are inserted into the microplate's spots, which also include alamar blue as a colorimetric indicator. A growth-positive control was also inoculated. The incubation was performed for 24 h at a temperature of 37 °C. The technique was applied and interpreted according to all the manufacturer's recommendations [16,17]. CLSI expert rules were applied to report the correct MIC value interpretation. The Sensititre Yeast-One is the eligible diagnostic method for defining yeast susceptibility profiles in our laboratory routine. All the MIC values gathered after the application of this method were confirmed with the CLSI broth microdilution method during the study. Therefore, the Sensititre Yeast-One technique was considered a reference during our investigations.

2.3. Micronaut-AM

The Micronaut-AM is a commercial microdilution method that includes anidulafungin (0.002–8 mg/L), micafungin (0.002–8 mg/L), caspofungin (0.002–8 mg/L), fluconazole (0.002–128 mg/L), posaconazole (0.008–8 mg/L), voriconazole (0.008–8 mg/L), itraconazole (0.03–4 mg/L), amphotericin B (0.03–16 mg/L), and 5-fluorocytosine (0.06–32 mg/L). All the antifungal drug dilutions are inserted into the microplate's spots, which are then implemented with methylene blue and an AST indicator to encourage the colorimetric reaction. A positive and a negative growth control were also evaluated on the same microplate. Particularly, the positive growth control was the first step to observe for a correct result interpretation. In the case of a light purple or blue color within the positive control spot after 24 h, the microplate was incubated for an additional 24 h (the incubation globally reached 48 h). According to this assumption, the incubation was generally performed for 24 h at a temperature of 37 °C, but *C. parapsilosis* isolates stayed for 48 h. The technique was applied and interpreted according to all the manufacturer's recommendations [18,19]. EUCAST expert rules were applied to report the correct MIC value interpretation.

2.4. Statistical Analysis

The collected data obtained from the microdilution methods were statistically analyzed using the Med-Calc Statistical Software version 17.9.2 (MedCalc Software bvba, Ostend, Belgium; <http://www.medcalc.org>; accessed on 5 May 2023). MIC₅₀ and MIC₉₀ were indicated for each *Candida* species and tested for antifungal drugs after the two methods, except for those with a restricted strain number, such as *C. glabrata*, *C. tropicalis*, and *C. krusei*. The essential agreement (EA) and the categorical agreement (CA) were also calculated where clinical breakpoints (CBP) and/or epidemiological cut-offs (E-COFF) were available. Specifically, the EA was defined within + one- or two-fold dilution. Otherwise, the CA was defined as the categorization agreement between the two applied methods. According to valuable published data [1], the absolute MIC values were considered, removing "<" and ">" signs from the calculations. CBP was used to classify *Candida* spp. as susceptible (S), intermediate (I), susceptible-dose dependent (SDD), or resistant (R) according to CLSI guidelines. Otherwise, clinical breakpoints were consulted to include *Candida* spp. as susceptible (S), susceptible-increased exposure (I), or resistant (R) according to EUCAST guidelines. An isolate belonging to the I or the SDD CLSI categories was included in the EUCAST I category, producing a categorical correspondence. E-COFFs are able to classify *Candida* spp. as wild-type (WT) or non-wild-type (non-WT) in the case of CBP indetermination. Isolates that belonged to S with one method and WT with the other method were defined as a categorical match, as were isolates that belonged to R with one technique and non-WT with the other one. The lack of a categorical match resulted in three error categories: minor errors, major errors, and very major errors. Minor errors were considered to be the presence of an isolate belonging to the I or SDD categories with Sensititre Yeast-One and S/WT (or R/non-WT) with Micronaut-AM. Major errors were defined in the case of isolates belonging to Sensititre Yeast-One S/WT but Micronaut-AM R/non-WT. Finally, very major errors appeared when isolates were classified as R/non-WT with Sensititre Yeast-One and S/WT with Micronaut-AM. These assumptions were applied according to the previously published data [1]. The simultaneous absence of CBP and E-COFFs led to the impossibility of obtaining a CA.

3. Results

All the positive samples originated from the peripheral blood of critical patients whose clinical history argued in favor of a candidaemia episode. The following species were identified: *Candida albicans* (20), *Candida parapsilosis* (18), *Candida tropicalis* (6), *Candida glabrata* sensu strictu (3), and *Candida krusei* (3). The MICs for *C. krusei* ATCC 6258 (8) and *C. parapsilosis* ATCC 22019 (8) were in the recommended ranges after the execution of both Sensititre Yeast-One and Micronaut-AM (Table 1).

Table 1. Quality control strains MIC ranges and modal MIC values.

| Quality Control Strain | Antifungal | Sensititre Yeast-One | | Micronaut-AM | |
|-----------------------------------|-----------------|----------------------|------------------|------------------|------------------|
| | | MIC Range (mg/L) | Modal MIC (mg/L) | MIC Range (mg/L) | Modal MIC (mg/L) |
| <i>C. krusei</i> ATCC 6258 | Anidulafungin | 0.06–0.12 | 0.12 | 0.06–0.125 | 0.06 |
| | Micafungin | 0.25–0.5 | 0.25 | 0.06 | 0.06 |
| | Caspofungin | 0.25–0.5 | 0.5 | 0.125–0.25 | 0.25 |
| | Fluconazole | 64 | 64 | 32 | 32 |
| | Posaconazole | 0.5 | 0.5 | 0.015–0.06 | 0.06 |
| | Voriconazole | 0.25–0.5 | 0.25 | 0.03–0.125 | 0.125 |
| | Itraconazole | 0.5 | 0.5 | 0.03–0.12 | 0.12 |
| | Amphotericin B | 0.5–1 | 1 | 0.25–0.5 | 0.5 |
| | 5-Fluorocytosin | 16 | 16 | 2–4 | 4 |
| <i>C. parapsilosis</i> ATCC 22019 | Anidulafungin | 0.25–1 | 0.5 | 0.12–0.25 | 0.12 |
| | Micafungin | 0.5 | 0.5 | 0.12 | 0.12 |
| | Caspofungin | 0.25–0.5 | 0.5 | 0.12–0.25 | 0.12 |
| | Fluconazole | 0.5–1 | 1 | 0.5–1 | 0.5 |
| | Posaconazole | 0.008–0.03 | 0.03 | 0.015–0.03 | 0.015 |
| | Voriconazole | 0.03 | 0.03 | 0.015–0.03 | 0.015 |
| | Itraconazole | 0.03–0.06 | 0.06 | 0.03 | 0.03 |
| | Amphotericin B | 0.25–1 | 0.25 | 0.125–0.25 | 0.125 |
| | 5-Fluorocytosin | 0.06 | 0.06 | 0.125 | 0.125 |

Table 2 summarizes the in vitro susceptibility data of all the identified *Candida* species after the application of the two tested methods. MIC₅₀ and MIC₉₀ values are indicated in the same table, along with MIC ranges for each tested antifungal drug.

Table 2. In vitro antifungal susceptibility of *Candida* spp. determined by Sensititre Yeast-One and Micronaut-AM.

| Antifungal Drugs | Species | Sensititre Yeast-One | | | Micronaut-AM | | |
|------------------|------------------------|----------------------|-------------------|-------------------|--------------|-------------------|-------------------|
| | | MIC Range | MIC ₅₀ | MIC ₉₀ | MIC Range | MIC ₅₀ | MIC ₉₀ |
| Fluconazole | <i>C. albicans</i> | 0.12–256 | 0.25 | 0.5 | 0.002–8 | 0.5 | 1 |
| | <i>C. glabrata</i> | 16–256 | NA | NA | 8–128 | NA | NA |
| | <i>C. parapsilosis</i> | 0.25–1 | 0.5 | 0.5 | 0.002–4 | 2 | 4 |
| | <i>C. tropicalis</i> | 1–8 | NA | NA | 0.5–2 | NA | NA |
| | <i>C. krusei</i> | 64–256 | NA | NA | NA | NA | NA |
| Posaconazole | <i>C. albicans</i> | 0.015–8 | 0.03 | 0.03 | 0.008–8 | 0.008 | 0.008 |
| | <i>C. glabrata</i> | 1–8 | NA | NA | 0.25–8 | NA | NA |
| | <i>C. parapsilosis</i> | 0.008–0.06 | 0.03 | 0.06 | 0.008–0.016 | 0.008 | 0.016 |
| | <i>C. tropicalis</i> | 0.008–1 | NA | NA | 0.008–0.03 | NA | NA |
| | <i>C. krusei</i> | NA | NA | NA | NA | NA | NA |

Table 2. Cont.

| Antifungal Drugs | Species | Sensititre Yeast-One | | | Micronaut-AM | | |
|------------------|------------------------|----------------------|-------------------|-------------------|--------------|-------------------|-------------------|
| | | MIC Range | MIC ₅₀ | MIC ₉₀ | MIC Range | MIC ₅₀ | MIC ₉₀ |
| Voriconazole | <i>C. albicans</i> | 0.008–8 | 0.008 | 0.015 | 0.008–8 | 0.008 | 0.008 |
| | <i>C. glabrata</i> | NA | NA | 8 | 0.125–1 | NA | NA |
| | <i>C. parapsilosis</i> | 0.008–0.5 | 0.015 | 0.015 | 0.008–0.03 | 0.016 | 0.03 |
| | <i>C. tropicalis</i> | NA | NA | 0.25 | 0.008–0.03 | NA | NA |
| | <i>C. krusei</i> | NA | NA | 1 | NA | NA | NA |
| Itraconazole | <i>C. albicans</i> | 0.03–16 | 0.06 | 0.12 | NA | 0.03 | 0.03 |
| | <i>C. glabrata</i> | NA | NA | 16 | 0.25–4 | NA | NA |
| | <i>C. parapsilosis</i> | 0.03–0.25 | 0.06 | 0.12 | 0.03–0.06 | 0.03 | 0.03 |
| | <i>C. tropicalis</i> | NA | NA | 0.25 | NA | NA | NA |
| | <i>C. krusei</i> | NA | NA | 0.5 | NA | NA | NA |
| Anidulafungin | <i>C. albicans</i> | 0.015–0.12 | 0.015 | 0.06 | 0.016–0.06 | 0.03 | 0.03 |
| | <i>C. glabrata</i> | NA | NA | 0.06 | 0.03–0.06 | NA | NA |
| | <i>C. parapsilosis</i> | 0.25–1 | 1 | 1 | 0.016–1 | 0.5 | 1 |
| | <i>C. tropicalis</i> | NA | NA | 0.25 | NA | NA | NA |
| | <i>C. krusei</i> | NA | NA | 0.12 | 0.25–0.5 | NA | NA |
| Micafungin | <i>C. albicans</i> | 0.008–2 | 0.015 | 0.015 | 0.03–0.25 | 0.03 | 0.03 |
| | <i>C. glabrata</i> | NA | NA | 0.03 | 0.016–0.06 | NA | NA |
| | <i>C. parapsilosis</i> | 0.5–2 | 1 | 1 | 0.016–0.25 | 0.25 | 0.25 |
| | <i>C. tropicalis</i> | NA | NA | 0.03 | 0.002–0.016 | NA | NA |
| | <i>C. krusei</i> | NA | NA | 0.25 | 0.06–0.125 | NA | NA |
| Caspofungin | <i>C. albicans</i> | 0.015–4 | 0.03 | 0.06 | 0.06–0.25 | 0.06 | 0.06 |
| | <i>C. glabrata</i> | NA | NA | 0.06 | 0.06–0.125 | NA | NA |
| | <i>C. parapsilosis</i> | 0.12–1 | 0.25 | 0.5 | 0.016–0.25 | 0.125 | 0.25 |
| | <i>C. tropicalis</i> | NA | NA | 0.06 | 0.003–0.125 | NA | NA |
| | <i>C. krusei</i> | NA | NA | 0.5 | NA | NA | NA |
| Amphotericin B | <i>C. albicans</i> | 0.12–1 | 0.5 | 0.5 | 0.03–0.125 | 0.125 | 0.25 |
| | <i>C. glabrata</i> | 0.12–0.25 | 0.12 | 0.25 | 0.125–0.5 | 0.5 | 0.5 |
| | <i>C. parapsilosis</i> | 0.12–0.5 | 0.25 | 0.25 | 0.03–0.5 | 0.5 | 0.5 |
| | <i>C. tropicalis</i> | 0.12–1 | 0.5 | 1 | 0.125–0.25 | 0.125 | 0.5 |
| | <i>C. krusei</i> | NA | 1 | 1 | 0.25–0.5 | 0.5 | 0.5 |
| 5-Fluorocytosin | <i>C. albicans</i> | 0.06–0.25 | 0.25 | 0.5 | 0.06–0.125 | 0.06 | 0.06 |
| | <i>C. glabrata</i> | NA | 0.06 | 0.06 | NA | 0.06 | 0.06 |
| | <i>C. parapsilosis</i> | 0.06–4 | 0.06 | 0.06 | 0.06–0.125 | 0.06 | 0.125 |
| | <i>C. tropicalis</i> | NA | 0.06 | 0.06 | NA | 0.06 | 0.06 |
| | <i>C. krusei</i> | 8–16 | 8 | 16 | 4–8 | 4 | 8 |

Abbreviations: NA, not available, which indicates the reporting of the same MIC value for all the tested isolates, with the impossibility to establish a value range, or the absence of a MIC₅₀/MIC₉₀ due to a restricted isolate number. MIC₅₀, the MIC value inhibiting the growth of >50% of isolates; MIC₉₀, the MIC value inhibiting the growth of >90% of isolates.

Furthermore, Table 3 schematized modal MIC values of all the tested species after the use of the two techniques.

Table 3. Modal MIC values of *Candida* spp. tested isolates after the application of two techniques.

| Species | Antifungal Drugs | Modal MIC (mg/L) | |
|------------------------|------------------|----------------------|--------------|
| | | Sensititre Yeast-One | Micronaut-AM |
| <i>C. albicans</i> | Anidulafungin | 0.015 | 0.016 |
| | Micafungin | 0.015 | 0.06 |
| | Caspofungin | 0.03 | 0.06 |
| | Fluconazole | 0.25 | 0.5 |
| | Posaconazole | 0.03 | 0.008 |
| | Voriconazole | 0.008 | 0.008 |
| | Itraconazole | 0.06 | 0.03 |
| | Amphotericin B | 0.5 | 0.125 |
| | 5-Fluorocytosin | 0.06 | 0.06 |
| <i>C. glabrata</i> | Anidulafungin | 0.015 | 0.03 |
| | Micafungin | 0.015 | 0.016 |
| | Caspofungin | 0.06 | 0.125 |
| | Fluconazole | NA | NA |
| | Posaconazole | 8 | 0.5 |
| | Voriconazole | NA | 0.125 |
| | Itraconazole | 16 | NA |
| | Amphotericin B | 0.12 | 0.5 |
| | 5-Fluorocytosin | 0.06 | 0.06 |
| <i>C. parapsilosis</i> | Anidulafungin | 1 | 1 |
| | Micafungin | 1 | 0.25 |
| | Caspofungin | 0.25 | 0.125 |
| | Fluconazole | 0.5 | 2 |
| | Posaconazole | 0.03 | 0.008 |
| | Voriconazole | 0.015 | 0.03 |
| | Itraconazole | 0.06 | 0.03 |
| | Amphotericin B | 0.25 | 0.5 |
| | 5-Fluorocytosin | 0.06 | 0.06 |
| <i>C. tropicalis</i> | Anidulafungin | 0.015 | 0.015 |
| | Micafungin | 0.03 | 0.03 |
| | Caspofungin | 0.06 | 0.06 |
| | Fluconazole | 1 | 1 |
| | Posaconazole | 0.25 | 0.25 |
| | Voriconazole | 0.06 | 0.06 |
| | Itraconazole | 0.25 | 0.25 |
| | Amphotericin B | 1 | 1 |
| | 5-Fluorocytosin | 0.06 | 0.06 |
| <i>C. krusei</i> | Anidulafungin | 0.12 | 0.015 |
| | Micafungin | 0.25 | 0.03 |
| | Caspofungin | 0.25 | 0.06 |
| | Fluconazole | NA | 1 |
| | Posaconazole | 0.5 | 0.25 |
| | Voriconazole | 0.5 | 0.06 |
| | Itraconazole | 0.5 | 0.25 |
| | Amphotericin B | 1 | 1 |
| | 5-Fluorocytosin | 0.06 | 8 |

Abbreviations: NA, Not applicable in the case of inhomogeneous MIC values distribution.

Notably, 1 *C. albicans* strain resulted in azole resistance after the application of both procedures. Specifically, the Sensititre Yeast-One method revealed a fluconazole MIC value of 256 mg/L and an itraconazole MIC value of 16 mg/L. Voriconazole and posaconazole

showed MIC values of 8 mg/L for the same isolate. The CLSI broth microdilution method confirmed these MIC values. The Micronaut-AM showed a MIC value of 8 mg/L for fluconazole, posaconazole, and voriconazole. Itraconazole revealed a susceptible MIC value of 0.03 mg/L, which was disproved by the EUCAST broth microdilution method (8 mg/L). A number of 1 *C. albicans* strains revealed pan-echinocandin resistance after the application of the two techniques. Precisely, a MIC value of 2 mg/L was reported for both micafungin and anidulafungin, while caspofungin revealed 4 mg/L after the Sensititre Yeast-One method. The CLSI broth microdilution method detected a MIC value of 4 mg/L for all the tested echinocandins, confirming the resistance. Regarding the same isolate, the Micronaut-AM reported a MIC value of 0.125 mg/L for anidulafungin and a MIC value of 0.25 mg/L for caspofungin and micafungin. The EUCAST broth microdilution method showed a MIC value of 0.25 mg/L for all the tested echinocandins, confirming the resistance detection. One *C. glabrata* isolate revealed a fluconazole resistance (MIC value = 256 mg/L) after the application of the Sensititre Yeast-One method, whose result was totally confirmed through the CLSI broth microdilution (MIC value = 256 mg/L). The Micronaut-AM technique on the same strains reported a resistance MIC value of 128 mg/L, which was confirmed through the EUCAST broth microdilution method (MIC value = 128 mg/L). One *C. tropicalis* isolate revealed fluconazole resistance after the application of the tested methods. Specifically, the Sensititre Yeast-One method reported a MIC value of 8 mg/L, which was confirmed through the CLSI broth microdilution method (MIC value = 8 mg/L). Otherwise, the Micronaut-AM technique revealed a MIC value of 2 mg/L, which was identically confirmed through the EUCAST broth microdilution method.

Regarding the essential agreement, *C. albicans* isolates reached a percentage of 100% for anidulafungin and 95% for micafungin (5% of discrepancies due to one strain whose MIC values differed more than two dilutions between the two methods). The same strains reported 90% EA for caspofungin (10% of discrepancies due to 2 isolates). A percentage of 90% of EA was also recorded by fluconazole (10% of discrepancies due to 2 isolates). A value of 95% was reached by posaconazole (5% of discrepancies due to 5 isolates). Voriconazole reported an EA of 100% (20% of discrepancies due to 4 isolates), as well as amphotericin. Finally, itraconazole and 5-fluorocytosin revealed an EA of 90% (10% of discrepancies due to 2 strains). *C. glabrata* isolates reported an EA of 100% for anidulafungin, caspofungin, micafungin, and amphotericin B. The same optimal result was reached by 5-fluorocytosin, posaconazole, and fluconazole. Unfortunately, a value of only 66.6% (33.3% of discrepancies due to one strain) was reported for both voriconazole and itraconazole. *C. parapsilosis* strains showed an EA of 100% for amphotericin and itraconazole. In addition, the EA value was 95% (5% of discrepancies due to one strain) for caspofungin, posaconazole, and voriconazole. A value of 83.3% (16.6% of discrepancies due to 3 isolates) was gathered for fluconazole and anidulafungin. 5-fluorocytosin revealed an 88.8% EA value (11.1% of discrepancies due to 2 isolates), while micafungin holds the lower record (77.7% of the EA value, with 22.2% of discrepancies due to 4 strains). *C. tropicalis* isolates revealed an EA of 100% for amphotericin B, anidulafungin, fluconazole, caspofungin, and 5-fluorocytosin. A value of 83.3% (16.6% of discrepancies due to 1 strain) was reported for itraconazole and micafungin. An EA percentage of 66.6% was recorded for voriconazole (33.3% of discrepancies due to 2 strains), while posaconazole showed an EA value of 50% (50% of discrepancies due to 3 strains).

Amphotericin, fluconazole, anidulafungin, caspofungin, posaconazole, itraconazole, voriconazole, and 5-fluorocytosin reached a 100% EA value in the case of *C. krusei* isolates. Micafungin reported an EA percentage of 66.6% (33.3% of discrepancies due to one isolate).

Regarding the categorical agreement, CLSI does not propose clinical breakpoints for some *Candida species* or amphotericin B. Despite this assumption, all the Sensititre Yeast-One often gathered MIC values lower than the CLSI E-COFF. In those cases, Micronaut-AM MIC values were all categorized as susceptible due to their inferiority over the CBP. As a consequence, there was a reliable match between the MIC collected after the application of the two investigated methods. According to these considerations, we decided to include

a CA calculation of 100% for some *Candida* species and amphotericin B. All the tested isolates showed susceptibility MIC values for caspofungin through Sensititre Yeast-One, except for one isolate. Micronaut-AM revealed comparable MIC values, but EUCAST did not provide any CBP or E-COFF. Despite this lack of reference, EUCAST guidelines suggest interpreting caspofungin in vitro susceptibility depending on the anidulafungin categorization of the analyzed isolate. According to this indication, we considered all the isolates susceptible to caspofungin through Micronaut-AM, except for one strain (which was also resistant to anidulafungin). Consequently, a CA of 100% can be hypothesized. As regards *C. tropicalis*, a CA percentage of 100% was reported for anidulafungin. CLSI allows the categorization of micafungin MIC values, but EUCAST guidelines only provide E-COFF. During the investigations, all the tested isolates showed caspofungin susceptibility through CLSI, maintaining MIC values under the EUCAST E-COFF value. Consequently, a 100% CA value was reported. All the tested isolates showed susceptibility MIC values for caspofungin through Sensititre Yeast-One. Micronaut-AM revealed comparable MIC values. EUCAST guidelines suggest interpreting caspofungin in vitro susceptibility depending on the anidulafungin categorization of the analyzed isolate. According to this indication, we considered all the isolates susceptible to caspofungin through Micronaut-AM. Consequently, a CA of 100% has been hypothesized. The EA and CA percentages for the tested antifungal drugs and the identified *Candida* species are summarized in Table 4.

Table 4. Essential and categorical agreement values between the Sensititre Yeast-One and Micronaut-AM for the different tested species.

| Antifungal Drugs | Isolates | | | | | | | | | | | |
|------------------|--------------------|------------------|--------------------|------|------------------------|------------------|----------------------|------------------|------------------|------------------|-------|------|
| | <i>C. albicans</i> | | <i>C. glabrata</i> | | <i>C. parapsilosis</i> | | <i>C. tropicalis</i> | | <i>C. krusei</i> | | Total | |
| | % EA | % CA | % EA | % CA | % EA | % CA | % EA | % CA | % EA | % CA | % EA | % CA |
| Anidulafungin | 100 ¹ | 100 | 100 | 100 | 83.3 | 100 | 100 ¹ | 100 | 100 | 66.6 | 94.0 | 98.0 |
| Micafungin | 95.0 | 100 | 100 | 100 | 77.7 | 100 | 83.3 | 100 | 66.6 | 100 | 76.0 | 100 |
| Caspofungin | 90.0 | 100 ² | 100 | 100 | 95.0 | 100 ² | 100 | 100 ² | 100 | 100 ² | 94.0 | 94.0 |
| Fluconazole | 90.0 | 100 | 100 | 33.3 | 83.3 | 88.8 | 100 | 94.4 | 100 | 100 | 82.0 | 88.0 |
| Posaconazole | 95.0 | NA | 100 | NA | 95.0 | NA | 50.0 | NA | 100 | NA | 64.0 | NA |
| Voriconazole | 100 | 100 | 66.6 | NA | 95.0 | 88.8 | 66.6 | 66.6 | 100 | 66.6 | 86.0 | 82.3 |
| Itraconazole | 90.0 | NA | 66.6 | NA | 100 | NA | 83.3 | NA | 100 | NA | 84.0 | NA |
| Amphotericin B | 100 | 100 ¹ | 100 ¹ | 100 | 100 | 100 ¹ | 100 | 100 | 100 | 100 ¹ | 100 | 100 |
| 5-fluorocytosin | 90.0 | NA | 100 | NA | 88.8 | NA | 100 | NA | 100 | NA | 92.0 | NA |

Abbreviations: EA, Essential agreement; CA, Categorical agreement; NA, Not applicable in the case of insufficient references by EUCAST and CLSI guidelines. ¹ *C. albicans* CA calculation for Amphotericin B was proposed due to the reliable match between Sensititre Yeast-One (MIC values lower than the E-COFF) and Micronaut-AM (MIC values categorized as susceptible); ² *C. albicans* CA calculation for caspofungin was proposed due to the comparable anidulafungin in vitro susceptibility, according to EUCAST suggestion.

4. Discussion

The increasing rates of invasive fungal infections and possible antifungal resistance extensively demonstrate the importance of testing antifungal susceptibility [8,20]. Regarding the most valuable testing method, EUCAST and CLSI committees have established standard guidelines [21,22]. They currently represent the reference methods to test antifungal susceptibility. Although their absolute precision and sensitivity are not known, several commercial versions, including Sensititre Yeast-One and the Micronaut-AM commercial microdilution methods, can satisfy this requirement [1]. The present study compared the effectiveness of these two techniques on *Candida* spp. These strains were immediately analyzed after identification, avoiding possible interferences or changes related to long storage. First, the experimental design allowed us to register some epidemiological data about *Candida* isolates features and diffusion. In our hospital setting, yeast strains are usually susceptible to the main antifungal drugs. Despite this observation, rare cases

of azole- or echinocandin-resistant *C. albicans* occurred among the collected strains. The resistance has been correctly detected by both the tested techniques, which were confirmed through EUCAST or CLSI standard microdilutions.

The comparison between this reference method and the Micronaut-AM results showed an optimal essential agreement rate for most antifungal drugs, especially for *C. albicans*. Voriconazole (*C. glabrata*, *C. tropicalis*), itraconazole (*C. glabrata*), posaconazole (*C. tropicalis*), and micafungin (*C. krusei*) occasionally showed low EA percentages, probably due to a restricted isolate number. Particularly, a single discrepant value highly impacted the global statistical evaluation because of the limited strains collected for some *Candida* species. In addition, a low EA value was reported for *C. parapsilosis* and micafungin. We hypothesize some difficulties in the growth of this yeast species, which required 48 incubation hours using Micronaut-AM. This method reported *C. parapsilosis* micafungin MIC values lower than the Sensititre Yeast-One technique. Thus, slight differences in agreement rates may also appear when reasoning in a species-specific sense, as demonstrated by similar published data [23].

The amphotericin B categorical agreement was homogeneously optimal for all the identified *Candida* species. All the echinocandins revealed elevated CA values for the tested isolates except for *C. krusei*. In our opinion, it is essential to perform further investigation into this species, including a higher isolate number. Globally, the high echinocandin CA should be considered an optimal result due to the difficulties in establishing breakpoint values for echinocandin, both from the EUCAST and CLSI committees [24].

The included *C. glabrata* isolates showed high fluconazole MIC values after the application of both methods. These results agree with the recent increase in fluconazole-resistant *C. glabrata* blood strains among countries around the world [25]. The *C. krusei* isolates confirmed moderately high voriconazole MIC values, which never suggested resistance episodes. The result matched literature data describing *C. krusei* isolates with voriconazole MIC values higher than those of *C. albicans* strains [26]. CA values were provided both for the association *C. glabrata*/fluconazole and the combination *C. krusei*/voriconazole. However, the restricted number of isolates included for these species suggests the need to plan further studies on this topic.

Furthermore, the restricted isolate number is the main reason why the fluconazole agreement rates differed from previous literature ones [1]. Particularly, we collected only 3 *C. glabrata* strains, while other studies involved a significantly higher number of isolates. This difference impacts CA and EA values, which deviate from previously reported data [1].

Voriconazole, 5-fluorocytosin, posaconazole, and itraconazole CA suffered from the impossibility of completing the calculation. Specifically, EUCAST and CLSI standard evidence should be produced to solve this profound lack of information. In addition, molecular diagnostic approaches should be considered an added value to the standard diagnostic workflow to investigate resistance or non-WT MIC values.

The experimental protocol also suffered from possible difficulties in interpretation. Specifically, azole MIC values are often marked by the trailing effect. It is described as persistent and reduced yeast growth within serial antifungal dilutions. The trailing effect could be related to the up-regulation of ergosterol genes. However, the inoculum precision and the incubation temperature could also be involved [21]. According to the gathered data, echinocandin MIC values generally report lower values after the Micronaut-AM application than after the execution of Sensititre Yeast-One. Furthermore, some *C. albicans* and *C. tropicalis* isolates show lower posaconazole MIC values when the Micronaut-AM method is applied. As a consequence, we hypothesized a slight MIC overestimation when these antifungal drugs were tested through the Sensititre Yeast-One method.

According to the available literature, Sensititre Yeast-One and Micronaut-AM are optimal commercial options to test antifungal susceptibility. Despite this assumption, it is difficult to produce a definitive comparison. On the one hand, only a few published studies are currently available about the Micronaut-AM technique, which has recently been investigated for mold susceptibility testing [27]. Most of the literary data [1,27] confirms the

reliability of the method for testing antifungal susceptibility. Moreover, Hitkova et al. [28] demonstrated the validity of this method in the epidemiological and microbiological surveillance of azole-resistant yeasts. On the other hand, Sensititre Yeast-One has been extensively consolidated in antifungal susceptibility testing [29,30]. Khumdee et al. [31] successfully applied this method to confirm the antifungal susceptibility of bloodstream yeast isolates, including uncommon *Candida* species. The results perfectly matched the standard microdilution method, confirming the high diagnostic value of this technique.

5. Conclusions

Obviously, it is not possible to establish which commercial microdilution method is better than the other one in defining the MIC for all the tested antifungal drugs against *Candida* spp. Sensititre Yeast-One has been calibrated according to the CLSI guidelines, while Micronaut-AM depends on EUCAST rules. As a result, the MIC result interpretation follows different principles. Both CLSI and EUCAST committees defined clinical breakpoints considering different information such as the distribution of MIC values, drug resistance episodes, pharmacological data, and clinical impacts. Although they took a similar approach, they established different methods to prepare the inoculum and the medium. According to these differences, breakpoints values may slightly change. The comparison between the two methods was performed only to enrich literary and laboratory data about two valuable techniques that appear to be essential alternatives to complete the diagnostic workflow in the case of invasive fungal infections. Furthermore, a molecular characterization of the detected resistant isolates could be interesting to investigate about the specific resistance mechanisms. In our opinion, future studies antifungal susceptibility testing should be encouraged, including possible clinical trials after the execution of both of the examined commercial microdilution methods.

The rationale should be the importance of investigating the clinical outcomes of critical patients after the therapeutical choices established on MIC values determined through both Sensititre Yeast-One and Micro-naut-AM.

Author Contributions: Conceptualization, L.T., M.C. and S.O.; methodology, L.T., S.O. and M.C.; investigation, L.T. and M.C.; data curation, L.T., M.C. and S.O.; writing—original draft preparation, L.T. and M.C.; writing—review and editing, S.O. and G.S.; supervision, L.T., G.S. and S.O.; All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: According to the applicable relevant national legislation, prior formal approval of the local Research Ethics Committee and informed consent were not mandatory, so they were not applied.

Data Availability Statement: All the research data are indicated within the present manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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