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Differential Sensitivity of the Species of *Candida parapsilosis* Sensu Lato Complex Against Statins

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Abstract *Candida parapsilosis* sensu stricto, *Candida orthopsilosis* and *Candida metapsilosis* are human fungal pathogens with clinical importance. The recently reclassified three closely related species have significant variation in virulence, clinical prevalence and susceptibility characteristics to different antifungal compounds. The aim of this study was to investigate the in vitro activity of atorvastatin and fluvastatin against *C. metapsilosis*, *C. orthopsilosis* and *C. parapsilosis*. Susceptibility tests showed that *C. parapsilosis* was the most sensitive while *C. orthopsilosis* was the least susceptible species to both drugs. On the basis of the differential sensitivity, we developed a simple, reliable and highly cost-effective plate assay to distinguish these closely related species. Applying this method, 54 isolates belonging to the *C. parapsilosis* sensu lato complex deposited in Szeged Microbial Collection could be sorted into the three species with 100 % probability.

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Introduction

Molecular studies of the *Candida parapsilosis* subtypes recently led to their separation into different, closely related species: the more prevalent *C. parapsilosis* sensu stricto, *Candida orthopsilosis* and *Candida metapsilosis* [1]. Human diseases caused by *C. parapsilosis* complex have significantly increased in importance and prevalence over the last decade [2, 3]. Recently, survey data from different hospitals [4, 5] show that *C. parapsilosis* sensu stricto is the second or third most common *Candida* spp. isolated from blood cultures. The examination of the distribution of the strains within the *C. parapsilosis* complex from different isolates obtained from blood and other sterile sources in European tertiary-care hospital revealed that the majority of the isolates (111 of 122) were *C. parapsilosis* sensu stricto, whereas significantly fewer (10 of 122) were identified as *C. orthopsilosis* and only one isolate was identified as *C. metapsilosis* [4]. This has been observed in other studies [5].

The sensitivity of the three species to antifungal drugs is different [4, 5], indicating that accurate identification would influence therapy. Conventional laboratory methods are unable to differentiate the

three species, and although molecular methods are rapid and sensitive, they are not routinely available.

Statins were originally identified as fungal metabolites [6] that act as selective inhibitors of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase [7], which is the enzyme responsible for the conversion of hydroxy-methylglutaryl-coenzyme A into mevalonic acid. In addition to their cholesterol-lowering effects, statins possess antifungal activity through inhibiting the formation of ergosterol, which is a key component of the cell membrane [8–10]. The minimal inhibitory concentrations of current statins against pathogenic fungi and yeasts are rather high; hence, they are not clinically useful as the required dose for a therapeutic effect would be associated with unacceptable serious, toxic side effects. However, statins can potentially serve to differentiate *Candida* species based on susceptibility profiles.

In the present study, the susceptibility of 13 strains of *C. metapsilosis*, 20 of *C. orthopsilosis* and 21 of *C. parapsilosis* sensu stricto to atorvastatin and fluvastatin was examined.

Materials and Methods

Yeast Strains and Cultivation

Strains used in these experiments are listed in Table 1. They were maintained on YPD (1 % glucose, 1 % peptone, 0.5 % yeast extract, 2 % agar) medium at 4 °C. Clinical isolates originated from Hungarian hospitals were identified by ITS sequencing in a previous work [11].

Statins

Atorvastatin (Atoris, KRKA) and fluvastatin (Lescol, Novartis) were of pharmaceutical grade. Stock solutions were prepared by dissolving the drugs in methanol and then stored at −80 °C. The concentration of the stock solutions was as follows: atorvastatin 20 mg mL^{-1}, and fluvastatin 40 mg mL^{-1}.

In Vitro Susceptibility Test

The in vitro antimycotic effect of atorvastatin and fluvastatin was determined by a microdilution method. The stock solution of statins was diluted in yeast nitrogen base (YNB) without amino acids (DIFCO) liquid medium and applied in final concentrations of 50–1.56 μg mL^{-1} in the case of atorvastatin and 25–0.78 μg mL^{-1} in the case of fluvastatin. The experiments were carried out in final volume of 100 μL in 96-well microplates. Yeast nitrogen base medium was prepared as suggested by the supplier and contained 1 % glucose as the carbon source. Yeast cell inoculates were prepared from 2-day-old cultures cultivated in YPD liquid medium at 30 °C with vigorous shaking. The cells were washed with sterile distilled water, and after repeated washing, they were suspended in YNB. The initial cell concentration in each well was adjusted to 4×10^4 cells mL^{-1}.

The microplate cultures were then grown at 30 °C for 48 h with shaking in a BIOTEK SynergyHT shaker. Growth was detected by checking the optical density (OD) of the cultures at 600 nm in every 5 min (Gen5 software). Non-inoculated medium was used as background, and the growth control contained inoculated YNB medium (initial cell concentration was 4×10^4 cells mL^{-1}). A growth control containing the same amount of methanol as the statin-containing samples was also applied.

The experiments were repeated two times, each plate carried two parallels per each strain and each statin.

Plate Assay

Strains cultivated for 48 h at 30 °C in YPD medium were diluted in YNB medium, and 10^5, 10^4, 10^3 and 10^2 cells were spotted onto YNB plates containing 30, 40 and 50 μg mL^{-1} fluvastatin or atorvastatin, respectively. The plates were incubated at 30 °C for 5 days.

Results and Discussion

The in vitro minimal inhibitory concentration (MIC) of atorvastatin and fluvastatin against the species of *C. parapsilosis* sensu lato complex was studied by microdilution method. Three strains of each species were included in the study: *C. metapsilosis* SZMC 8022, SZMC 1547, SZMC 1548; *C. orthopsilosis* SZMC 1545, SZMC 8119, SZMC 8121; and *C. parapsilosis* CBS 6318, SZMC 1577, SZMC 8002.

Table 1 *Candida* isolates

	Species	Strain no.	Isolation place
1	*C. parapsilosis*	SZMC 1361	Blood, Debrecen, Hungary
2	*C. parapsilosis*	SZMC 1438	Blood, Debrecen, Hungary
3	*C. parapsilosis*	SZMC 8043C	Stomach, Pécs, Hungary
4	*C. parapsilosis*	SZMC 8045	Blood, Pécs, Hungary
5	*C. parapsilosis*	SZMC 1577	Szeged, Hungary
6	*C. parapsilosis*	SZMC 1572	Szeged, Hungary
7	*C. parapsilosis*	SZMC 1568	Szeged, Hungary
8	*C. parapsilosis*	SZMC 8050C	Szeged, Hungary
9	*C. parapsilosis*	SZMC 1587	Szeged, Hungary
10	*C. parapsilosis*	SZMC 8051C	Szeged, Hungary
11	*C. parapsilosis*	SZMC 1569	Szeged, Hungary
12	*C. parapsilosis*	SZMC 8004	Ear, Debrecen, Hungary
13	*C. parapsilosis*	SZMC 1594	Cannula, Debrecen, Hungary
14	*C. parapsilosis*	SZMC 1590	Urine, Debrecen, Hungary
15	*C. parapsilosis*	SZMC 1592	Sputum, Debrecen, Hungary
16	*C. parapsilosis*	SZMC 8002	Ear, Debrecen, Hungary
17	*C. parapsilosis*	SZMC 1596	Wound, Debrecen, Hungary
18	*C. parapsilosis*	CBS 1954	Olive, Italy
19	*C. parapsilosis*	CBS 6318	Olive
20	*C. parapsilosis*	SZMC 8112	Germany
21	*C. parapsilosis*	SZMC 8113	Sputum, Cagliari, Italy
22	*C. metapsilosis*	SZMC 8029	Blood, Debrecen, Hungary
23	*C. metapsilosis*	SZMC 1547	Unknown
24	*C. metapsilosis*	SZMC 1548	Unknown
25	*C. metapsilosis*	SZMC 8022	Throat, Pécs, Hungary
26	*C. metapsilosis*	SZMC 8091	Sputum, Pisa, Italy
27	*C. metapsilosis*	SZMC 8092	Bronchial aspirate, Pisa, Italy
28	*C. metapsilosis*	SZMC 8093	Nail, Pisa, Italy
29	*C. metapsilosis*	SZMC 8094	Feces, Pisa, Italy
30	*C. metapsilosis*	SZMC 8095	Nail, Pisa, Italy
31	*C. metapsilosis*	SZMC 8096	Nail, Pisa, Italy
32	*C. metapsilosis*	SZMC 8097	Feces, Pisa, Italy
33	*C. metapsilosis*	SZMC 8098	Feces, Pisa, Italy
34	*C. metapsilosis*	SZMC 8099	Peripheral blood, Auckland, New Zealand
35	*C. orthopsilosis*	SZMC 1545	Unknown
36	*C. orthopsilosis*	SZMC 1546	Unknown
37	*C. orthopsilosis*	SZMC 8115	Germany
38	*C. orthopsilosis*	SZMC 8116	Germany
39	*C. orthopsilosis*	SZMC 8117	Germany
40	*C. orthopsilosis*	SZMC 8118	Germany
41	*C. orthopsilosis*	SZMC 8119	Germany
42	*C. orthopsilosis*	SZMC 8120	Germany
43	*C. orthopsilosis*	SZMC 8121	Germany
44	*C. orthopsilosis*	SZMC 8122	Germany
45	*C. orthopsilosis*	SZMC 8100	Pisa, Italy, nail

Table 1 continued

	Species	Strain no.	Isolation place
46	*C. orthopsilosis*	SZMC 8101	Pisa, Italy, skin
47	*C. orthopsilosis*	SZMC 8102	L' Aquila, Italy, catheter
48	*C. orthopsilosis*	SZMC 8103	Pisa, Italy, bronchial aspirate
49	*C. orthopsilosis*	SZMC 8104	Pisa, Italy, nail
50	*C. orthopsilosis*	SZMC 8105	Pisa, Italy, bronchial aspirate
51	*C. orthopsilosis*	SZMC 8106	NCPF, UK, unknown
52	*C. orthopsilosis*	SZMC 8107	Pisa, Italy, skin
53	*C. orthopsilosis*	SZMC 8108	Pisa, Italy, sputum
54	*C. orthopsilosis*	SZMC 8109	Pisa, Italy, catheter

The applied concentration range was between 50 and 1.56 μg mL^{-1} for atorvastatin (Fig. 1) and 25–0.78 μg mL^{-1} for fluvastatin (Fig. 2). The antifungal activity of the atorvastatin varied; *C. parapsilosis* was the most sensitive species while the susceptibility of *C. orthopsilosis* and *C. metapsilosis* was similar (Fig. 1). The MIC_{100} value (100 % growth inhibition) of atorvastatin was 25 μg mL^{-1} for *C. parapsilosis*

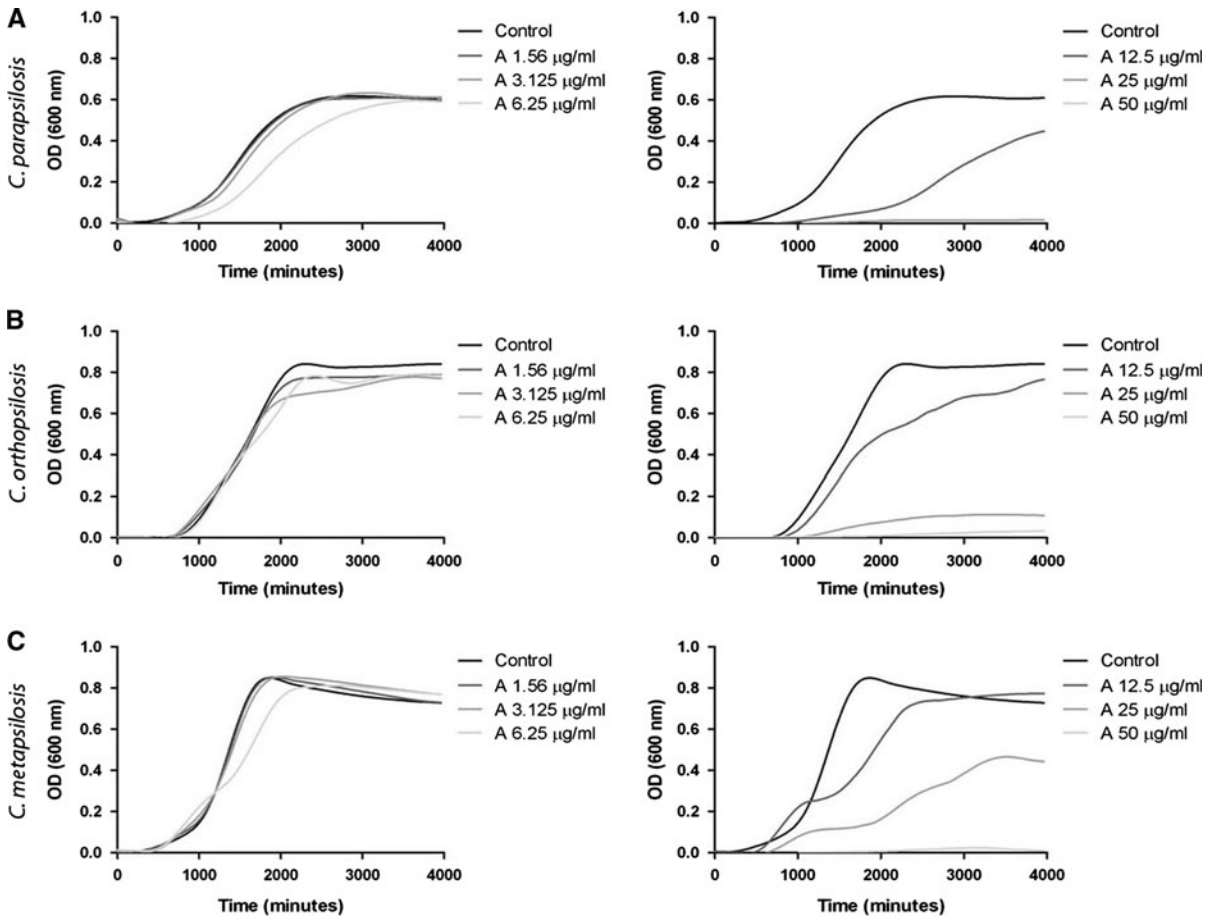

Fig. 1 Growth *curves* of three representative isolates of *C. parapsilosis* (**a**), *C. orthopsilosis* (**b**) and *C. metapsilosis* (**c**) in the presence of different concentrations of atorvastatin, measured in 5-min intervals for 66 h

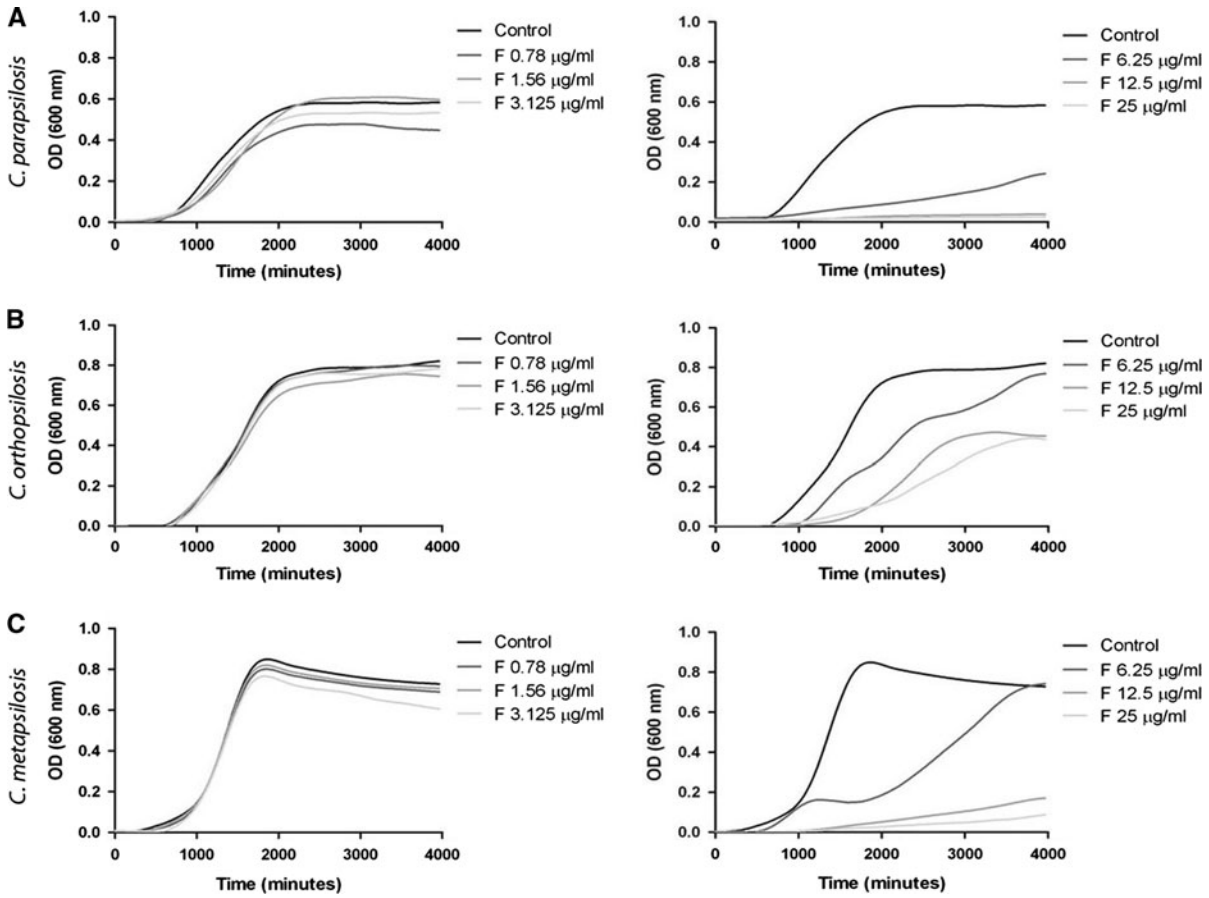

Fig. 2 Growth *curves* of three representative isolates of *C. parapsilosis* (**a**), *C. orthopsilosis* (**b**) and *C. metapsilosis* (**c**) in the presence of different concentrations of fluvastatin, measured in 5-min intervals for 66 h

(Fig. 1a) and 50 μg mL^{-1} for *C. orthopsilosis* and *C. metapsilosis* (Fig. 1b, c).

Remarkable differences were detected in the sensitivity to fluvastatin: *C. parapsilosis* was the most susceptible species, while *C. orthopsilosis* proved the less sensitive to it (Fig. 2). Fluvastatin was more effective than atorvastatin, as it completely inhibited the growth of *C. parapsilosis* at 12.5 μg mL^{-1} concentration (Fig. 2a). *C. orthopsilosis* was the least susceptible to fluvastatin (Fig. 2b) as 12.5 μg mL^{-1} concentration caused only 50 % growth inhibition. In the case of *C. metapsilosis*, 90 % inhibition was demonstrated at this concentration (Fig. 2c).

On the basis of the data presented above, we tested the sensitivity of our strains of the three species on solidified YNB medium supplemented with 30, 40 and 50 μg mL^{-1} fluvastatin or atorvastatin. After 5 days of incubation at 30 °C, none of the 21 strains of *C. parapsilosis* sensu stricto could grow on medium containing the lowest concentration, 30 μg mL^{-1}, of either fluvastatin or atorvastatin. In contrast, the 20 strains of *C. orthopsilosis* and the 13 strains of *C. metapsilosis* formed colonies at 50 μg mL^{-1} of atorvastatin. At 30 μg mL^{-1} of fluvastatin, *C. orthopsilosis* cells grew with inoculates as low as 10^2 cells (Fig. 3b), whereas *C. metapsilosis* colonies were detected only at the highest cell densities (Fig. 3c). Hence, fluvastatin at 30 μg mL^{-1} concentration was sufficient to discriminate between *C. metapsilosis* and *C. orthopsilosis* using different inoculums of these species. Based on these results, fluvastatin was significantly more discriminatory than atorvastatin.

Species differentiation is laborious in the case of closely related human pathogenic fungi or yeasts. Several conventional and molecular methods exist, but all of them have their limits, so new reliable

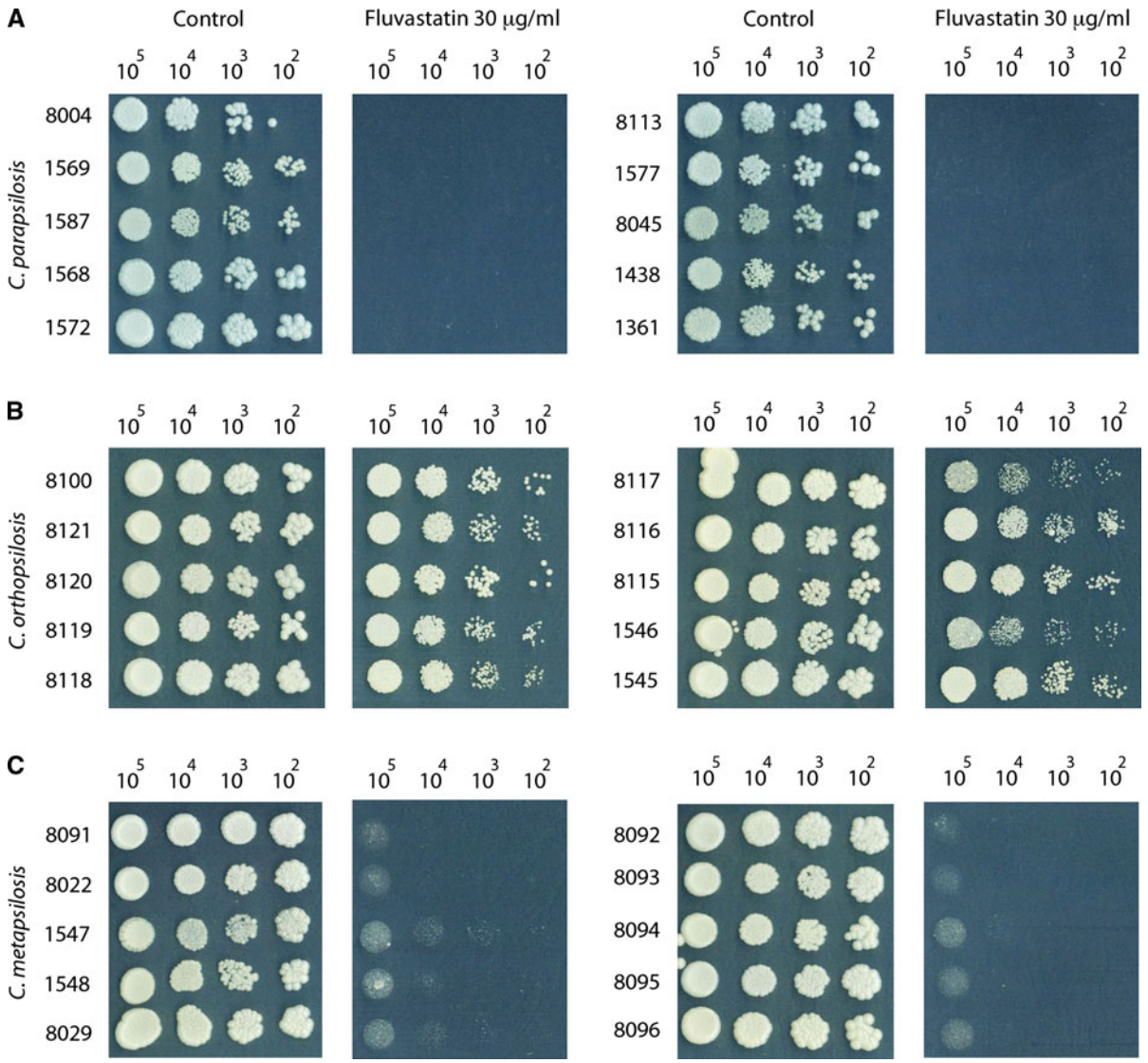

Fig. 3 Colony formation of ten representative strains of *C. parapsilosis* (**a**), *C. orthopsilosis* (**b**) and *C. metapsilosis* (**c**) on *solid* YNB medium (control) and YNB supplemented with 30 µg mL^{-1} fluvastatin

approaches are required. Differences in lovastatin sensitivity have been used for species selection in *Rhizomucor* genus in a recent study [12]. Based on the remarkable difference observed in the susceptibilities of *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis* to fluvastatin, we propose a simple plate assay to distinguish the strains of the three species. Our data indicate that a dilution series of the isolated strain should be inoculated on YNB medium containing 30 µg mL^{-1} fluvastatin. *C. parapsilosis* strains should not grow under this condition (Fig. 3a). As *C. orthopsilosis* is the most resistant species to this drug, robust growth should occur at each dilution (Fig. 3b), whereas only the dilutions with 10^4 or 10^5 of *C. metapsilosis* will have growth (Fig. 3c). The method was validated with 13 strains of *C. metapsilosis*, 20 *C. orthopsilosis* and 21 *C. parapsilosis* sensu stricto. In conclusion, this method provides a simple and effective method for differentiating these three closely related species, and this approach can be used as an alternative technique for definite identification of *C. parapsilosis* sensu lato isolates, which will be a useful tool for future epidemiological studies.

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