Original article JAC

Antifungal susceptibility profiles of rare ascomycetous yeasts

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Objective: The objective of the study was to generate antifungal susceptibly patterns for *Trichomonascus ciferrii* (*Candida ciferrii), Candida inconspicua* (*Torulopsis inconspicua*), and *Diutina rugosa* species complex (*Candida rugosa* species complex), and to provide key parameters such as MIC_{50}, MIC_{90}, and epidemiological cut-off values (ECOFFs).

Method: Our strain set included *Candida inconspicua* (n=168), *D. rugosa* species complex (n=90) *(D. pararugosa* (n=60), *D. rugosa* (n=26), and *Candida mesorugosa* (n=4)), *Pichia norvegensis* (*Candida norvegensis*) (n=15), and *C. ciferrii* (n=8). Identification was performed by MALDI-TOF MS or internal transcribed spacer sequencing. Antifungal susceptibility patterns were generated for azoles, echinocandins, and amphotericin B using commercial Etest® and EUCAST broth microdilution method v7.3.1. Essential and categorical agreement was calculated for Etest® and EUCAST.

Results: *C. inconspicua* and *P. norvegensis* showed elevated MICs towards azoles (MIC_{50} ≥0.125 mg/L). *D. rugosa* and *C. pararugosa* presented high MICs towards echinocandins (MIC_{50} ≥0.06 mg/L). Agreement between methods was generally low (<90%), essential agreement averaged 70%, and categorical agreement was lower with an average of 55%. ECOFFs were suggested for *C. inconspicua* and *D. rugosa* species complex.

Conclusion: Rare yeast species tested shared high fluconazole MICs. *D. rugosa* species complex displayed high MICs for echinocandins, while *C. inconspicua* and *P. norvegensis* were found to have high MICs for triazoles. Overall, the agreement between EUCAST and Etest® was poor and therefore MIC values generated with Etest® cannot be directly compared with EUCAST results.

Introduction

Ascomycetous yeasts such as *Candida albicans* cause a large number of infections every year and are a major public health concern world wide[1]. Therefore, considerable efforts and resources have been used to study the most common *Candida* species such as *C. albicans* and *Candida glabrata*[2, 3]. The genus *Candida* represents an artificial morphological genus that is highly polyphyletic[4]. The term rare yeast accounts for a group of ascomycetous yeasts with a prevalence of ≤1% in clinical *Candida* infections[2]. Members of this artificial group are phylogenetically distantly related, and are characterized by their elevated MICs (minimal inhibitory concentration) to at least one class of antifungals[5]. Some species were renamed due to changes in the "International code of nomenclature for algae, fungi and plants" (Melbourne code)[4] such as *Diutina rugosa* (synonym: *Candida rugosa)*, *Candida pararugosa*, *Trichomonascus ciferrii* (synonym: *Candida ciferrii), Candida inconspicua (basionym: Torulopsis inconspicua),* and *Pichia norvegensis (*synonym*: Candida norvegensis)* among others. The linkage between phylogenetic relation and antifungal susceptibility pattern (intrinsic resistance) was previously shown[6].

Only few MIC data for ascomycetous yeasts are available and are based on a limited number of clinical studies or case reports[7]. In addition, sibling or cryptic species can only be identified by internal transcribed spacer (ITS) sequencing or using MALDI-TOF MS[8]. Infections by rare fungal pathogens are linked to high mortality and therapeutic failure[7, 9-11]. Recently *Candida auris,* a rare emerging yeast[12], caused outbreaks in American[13], Indian[14], British[15] and Spanish[16] hospitals that were particularly difficult to control due to multi-drug resistance and limited clinical experience[5]. Epidemiological cut off values (ECOFF) and clinical breakpoints (CBP) for the international standard methods EUCAST[13] and CLSI[14] have not yet been established for the majority of antifungal drugs and rare yeast species. Few case reports point to elevated MICs for at least one azole or for whole drug classes, such as in *T. ciferrii*[2], *Candida guilliermondii*[15], *Candida haemulonii*[16], *C. inconspicua*[2, 4, 17], and *P. norvegensis*[2, 4, 17]. Echinocandin resistance was described in *T. ciferrii*[18].

The aim of this study was to generate data on antifungal susceptibility patterns for *C. inconspicua*, *D. rugosa* species complex, and *T. ciferrii* and to determine ECOFFs for posaconazole (POS), isavuconazole (ISA), itraconazole (ITC), fluconazole (FLC), voriconazole (VRC), micafungin (MICA), caspofungin (CAS), anidulafungin (ANI), and amphotericin B (AMB) with respect to the number of the strains tested for that particular species. In addition, we compared MICs generated by using EUCAST broth-microdilution method[17] with those by Etest®.

Material and methods

Strain collection

Clinical isolates of *D. rugosa* species complex, *C. inconspicua*, or *T. ciferrii* were sent by members of ISHAM (The International Society for Human & Animal Mycology), EFISG (European Fungal Infection Study Group), or ECMM (European Confederation of Medical Mycology), here designated as "Rare Yeast Study Group", to the Division of Hygiene and Medical Microbiology (HMM) of the Medical University of Innsbruck. Species identification was confirmed at the HMM by MALDI-TOF MS and ITS sequencing. The final strain collection comprised 281 strains from 13 different countries and 26 institutions.

Identification

All isolates were grown on Sabouraud's 2% dextrose agar at 30ºC for 72h upon arrival and identified using MALDI-TOF mass spectrometry (MALDI-Biotyper, Bruker, Daltonics, Database version, USA). Strains that had inconclusive results in the MALDI-TOF MS identification were identified by sequencing of the ITS region[18] (Table S2).

Antifungal susceptibility testing

Etest. Etest® strips for POS, ISA, ITC, FLC, VRC, MICA, CAS, ANI, and AMB (all bioMérieux SA, France, except ISA, which was provided by Liofilchem, Roseto degli Abruzzi, Italy) were performed according to the manufacturers' instructions. Hereafter, the term Etest® includes both bioMérieux and Liofilchem test-strips. Plates were incubated at 37 °C and visually read after 48h. MIC values were rounded to the next higher dilution step of EUCAST for method comparison.

EUCAST. Broth-microdilution was performed according to EUCAST guidelines[17] with the following minor modifications due to slow growth of the rare yeast: (1) incubation time was prolonged to 48h, and (2) OD (optical density) threshold of plate reader reading was lowered to 0.1. Due to the lowering of thresholds, MICs were read with plate reader and by visual reading for comparison.

POS (Schering-Plough, New Jersey, USA), ISA (Basilea, Basel, Switzerland), ITC (Sigma, Rowville, Australia), FLC (Sigma), VRC (Sigma), MICA (Astellas, Munich, Germany), CAS (Sigma), ANI (Pfizer, New York, USA), and AMB (Sigma). Plates (Cellstar Cat-No. 655180, Greiner Bio-One, USA) were evaluated at 48h both visually and by plate reader (Microplate Reader model 680, Bio-Rad, USA). *Candida parapsilosis* ATCC 22019 or *Candida krusei* ATCC 6258 were used as quality control. MIC range, MIC_{50}, and MIC_{90} were calculated for each species when testing ≥ 15 isolates. ECOFFs were set from the central value of a Gaussian distribution[19, 20] for *D. rugosa* species complex (n=90) and *C. inconspicua* (n=168).

Agreement rates between EUCAST and Etest®

Essential agreement was defined as ±2 fold dilution variation between the methods[21, 22]. Categorical agreement was considered to be achieved if the strain was categorized equally by both methods, when CBPs of *C. albicans* were used for categorization. In the absence of any cut-off values, CBPs for *C. albicans* were used for MIC comparison studies. Hence, some statements may be considerably limited.

Categorical changes between adjacent categories were considered a minor error, while a major error was attributed to a change in category that skips from susceptible to resistant and vice versa[21, 22].

Results

Susceptibility testing

281 clinical isolates comprising *C. inconspicua* (n=168), *D. rugosa* species complex (n= 90) (*C. pararugosa* (n=60), *D. rugosa* (n=26) and *C. mesorugosa* (n=4)), *P. norvegensis* (*C. norvegensis*) (n=15), and *C. ciferrii* (n=8) were investigated.

Etest. On average Etest® showed high MICs for all tested antifungals when compared to CBPs of *C. albicans* (Table S3). Specifically, *C. inconspicua* and *P. norvegensis* showed distinctly higher MIC values for the whole group of azoles (≥0.125 mg/L), *D. rugosa* and *C. pararugosa* had increased MIC values towards echinocandins (ANI, MICA, and CAS) of ≥0.06 mg/L. Overall, ISA was the most effective azole, while ANI was the most effective echinocandin. AMB exhibited good activity against all species. Etest® values were consistently equal or lower for echinocandins and for AMB than the values obtained by EUCAST. The MIC_{50} and MIC_{90} of Etest® were similar to those of EUCAST and essential agreement was achieved by the majority (Table. 1). We propose for *C. inconspicua* the following ECOFFs for Etest® for non-wildtype strains: AMB ≥ 0.25 mg/L, ANI ≥ 0.06 mg/L, CAS ≥ 4.0 mg/L, FLC >64.0 mg/L, ISA ≥ 1.0 mg/L, ITC ≥ 4.0 mg/L, MICA ≥ 0.25 mg/L, POS ≥ 2.0 mg/L and VRC ≥ 1.0 mg/L (Table 1), for *D. rugosa* species complex AMB ≥ 1.0 mg/L, ANI ≥ 1.0 mg/L, CAS ≥ 4.0 mg/L, FLC ≥ 32.0 mg/L, ISA ≥ 0.25 mg/L, ITC ≥ 1.0 mg/L, MICA ≥ 1.0 mg/L, POS ≥ 0.25 mg/L and VRC ≥ 0.5 mg/L (Table 1).

EUCAST. EUCAST and Etests® showed the same trend of high MICs for the most common species in our collection; MIC_{50} in echinocandins and azoles were equal or higher than the CBPs of *C. albicans*[23]. *C. inconspicua* and *P. norvegensis* displayed high MIC_{50} values for azoles (≥0.25 mg/L for ITC, ≥0.125 mg/L for POS, ≥0.125 mg/L for ISA, ≥32 mg/L for FLC, and ≥0.125 mg/L for VRC) in both, EUCAST and Etest®. *D. rugosa* and *C. pararugosa* exhibited high MICs against echinocandins: ≥0.06 mg/L ANI,

≥0.125 mg/L MICA, and ≥0.5 mg/L CAS for both EUCAST and Etest®. Azole MICs exceeded the *C. albicans* resistance CBPs. The MIC_{50} and MIC_{90} are in most cases within the essential agreement pointing to a compact MIC distribution of the tested strain population. ECOFFs were on average several dilutions above the CBPs of *C. albicans*, not knowing the clinical value of this finding. In addition, ECOFFs were higher than the MIC_{90} indicating a narrow MIC distribution of the population (Table 1). In general, the majority of species displayed high MIC values for at least one antifungal class and for *T. ciferrii* was valid for both, azoles and echinocandins. We propose for *C. inconspicua* the following ECOFFs for EUCAST (non-wildtype): AMB >4.0 mg/L, ANI ≥ 0.25 mg/L, CAS >4.0 mg/L, FLC >64.0 mg/L, ISA ≥ 2.0 mg/L, ITC ≥ 2.0 mg/L, MICA ≥ 0.5 mg/L, POS ≥ 2.0 mg/L and VRC ≥ 2.0 mg/L (Table 1). For *D. rugosa* species complex AMB ≥ 1 mg/L, ANI > 4.0 mg/L, CAS > 4.0 mg/L, FLC ≥ 64.0 mg/L, ISA ≥ 4.0 mg/L, ITC ≥ 4.0 mg/L, MICA >4.0 mg/L, POS ≥ 2.0 mg/L and VRC ≥ 2.0 mg/L (Table 1).

In our study, *C. pararugosa* was the most common species of the *D. rugosa* species complex, followed by *D. rugosa*. For *C. mesorugosa* only four isolates were available. For ECOFF calculation all sibling species within the *D. rugosa* species complex were pooled, as MIC_{50} and MIC_{90} values were similar for all species varying by a maximum of two dilution steps.

Agreement

Essential agreement between Etest® and EUCAST was low (average 79.56%), and varied greatly (23-100%). FLC was the antifungal drug with the best essential agreement with an average of 85.4%. *C. inconspicua* and *C. pararugosa* showed lowest essential agreement with AMB, displaying 29.7% and 38.9%, respectively. *D. rugosa* wasfound to show lowest essential agreement for ANI (23%). Lacking comparison values, categorical agreement was based on CBPs of *C. albicans* and was found to be a drug- and species-dependent feature. Overall, highest categorical agreement was achieved for FLU in *C. inconspicua* (95%) and ANI for the *D. rugosa* species complex (72%), lowest for VRC with *C. inconspicua* (42%) and *C. pararugosa* (17%), for FLC with *D. rugosa* (35%) and for ANI with *P. norvegensis* (53%). EUCAST reading visually and by plate reader had the same tendencies though values

varied slightly; however, values were within essential agreement (Table 2). Variations of >2 two-fold dilution steps were observed in *D. rugosa* species complex for the azoles and AMB. ECOFFs were always equal or higher for Etest® than for EUCAST method. Minor differences have been observed for visually EUCAST reading results compared to the results generated with the plate reader (Table 1).

Discussion

A number of clinically rare species within the *Candida* genus form distinct species complexes. The *D. rugosa* species complex consists of *D. rugosa sensu stricto*, *C. pseudorugosa*, *C. neorugosa*, *C. mesorugosa*, and *C. pararugosa*[24]. Among the species tested in our study, *C. inconspicua*, *D. rugosa*, and *C. pararugosa* had high MIC values for FLC which is consistent with literature[2, 5, 9, 25-31]. *P. norvegensis* and *C. krusei* are phylogenetically related[32] and have similar susceptibility patterns as *C. inconspicua* with high MIC values for FLC, ITC, and POS which are also reported in literature[2, 9, 29]. *T. ciferrii* is also known to be FLC resistant[2, 25]. Direct comparison is difficult due to the lack of ECOFFs and CBPs[17, 33]. *C. inconspicua* and *P. norvegensis* displayed a higher degree of resistance towards azoles sharing $MIC_{50} \geq 0.125$ mg/L. Testing a limited strains set[5], *D. rugosa* and *C. pararugosa* expressed high MICs for all echinocandins (Table 1). *D. rugosa* is known to be FLC resistant[5], but for *C. pararugosa* we report this for the first time.

EUCAST broth microdilution method is an internationally standardized method and therefore easily comparable with objective endpoints due to photometric reading. However, the incubation temperature combined with the medium composition of RPMI seems to be suboptimal for the growth of some rare species, resulting in slow growth[34]. Our and other's data showed that an increase in incubation time only causes a minor increase of MIC values[35]. However, for the rare species, growth after 48 h of incubation was lower in absolute optical density than growth of *C. albicans* after 24 h incubation, suggesting that high MIC values are not due to a strong growth after 48 h incubation. Method variability between EUCAST and Etest® was found previously[36-41], a major difference between EUCAST[17] and CLSI[33] CBPs exist for the echinocandin class. In our study, the agreement was low (<90%) between EUCAST and

Etest®. The low agreement was nevertheless neither fully species- nor drug-specific, which point to a mixture of biological and chemical factors. Categorical agreement was also low (<90%), and was mostly dependent on the polarity of the distribution (Table 2).

Categorical and essential agreements do not correlate, as the former depends on the distribution of the population with respect to CBPs. For example in the case of MICA, *C. pararugosa* had a low essential agreement between EUCAST and Etest®, but good categorical agreement. In contrast *C. inconspicua* showed a good essential agreement, but a low categorical agreement. The lowest overall agreement was for *C. pararugosa* (essential agreement 39%-70% and categorical agreement 17%-97%) (Table 2). Essential agreement between the methods is low and from a clinical perspective the categorical agreement is also low, therefore Etest® results need to be evaluated with caution when compared with EUCAST. Discrepancies between the methods are difficult to explain and may have multiple causes comprising mainly methodical differences such as agar versus broth based techniques, including different endpoint reading definitions.

The clinical need of the differentiation of the sibling species within the *D. rugosa* species complex remains unknown. For *C. mesorugosa* only few isolates were available to study, while *D. pseudorugosa*, *D. neorugosa* were absent from our strain collection. *Candida pararugosa* and *C. mesorugosa* had both high MICs ≥64 µg/mL for FLC using EUCAST standard method. Also high MICs against echinocandins were a common feature of *C. pararugosa* and *D. rugosa*. Species studied may carry intrinsic resistance against certain drugs, since the whole populations expressed high MICs. The resistance patterns also correlated with phylogenetically related species, which is in line with previous studies[6]. For example, *C. inconspicua*, *P. norvegensis*, and *C. krusei* have all high azole MIC patterns[2, 9, 29] and are related phylogenetically[32]. *D. rugosa* and *C. pararugosa* are closely related[42] and have high resistance levels to echinocandins. All this could be potentially an indicator that these characteristics may be inherited from a common ancestor.

Conclusion

Species within the *D. rugosa* species complex (*D. rugosa*, *C. pararugosa*, and *C. mesorugosa*) exhibit similar resistance patterns with high MICs for FLC and the echinocandins. *Candida inconspicua* and *P. norvegensis* have similar resistance patterns with high MIC values towards triazoles. The agreement between EUCAST and Etest® is poor and therefore MIC values generated with Etest® are not directly comparable to EUCAST results.

Acknowledgements

To Katherina Rosam for her help and incalculable technical support (Division of Hygiene and Medical Microbiology, Medical University of Innsbruck).

Funding

This project received funding from the European Union´s Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement (No 642095).

Conflict of interest

A. Pérez-Hansen has no potential conflicts of interest to declare.
M. Lackner has no potential conflicts of interest to declare.
C. Lass-Flörl has received grant support from the Austrian Science Fund (FWF), MFF Tirol, Astellas Pharma, Gilead Sciences, Pfizer, Schering Plough, and Merck Sharp & Dohme. She has been an advisor/consultant to Gilead Sciences, Merck Sharp & Dohme, Pfizer, and Schering Plough. She has received travel/accommodation expenses from Gilead Sciences, Merck Sharp & Dohme, Pfizer, Astellas, and Schering Plough and has been paid for talks on behalf of Gilead Sciences, Merck Sharp & Dohme, Pfizer, Astellas, and Schering Plough.

M. Aigner has received travel grants, honorarium as a speaker and consultancy fee from Astellas Pharma, MSD, and Gilead.

A. Alastruey-Izquierdo, in the past 5 years, has received research grants from ESCMID, ECMM, Fondo de Investigaciones Sanitarias, Gilead Sciences, F2G, and Scynexis. She has been an advisor for Gilead Science. She has been paid for talks on behalf of Gilead Sciences, Merck Sharp and Dohme and Pfizer.

S. Arikan-Akdagli does not have any potential conflict of interest related particularly to this paper. Otherwise, she has received investigator initiated research grant support from Pfizer and speaker honoraria or travel grant from Astellas, Gilead, and Pfizer in the past five years.

O. Bader has no potential conflicts of interest to declare.

K. Becker has no potential conflicts of interest to declare.

T. Boekhout has no potential conflicts of interest to declare.

W.Buzina has no potential conflicts of interest to declare.

O.A. Cornely received research grants from Amplyx, Astellas, Basilea, Cidara, F2G, Gilead, Matinas, Merck/MSD, Pfizer, Scynexis, is a consultant to Amplyx, Astellas, Basilea, Cidara, F2G, Gilead, Matinas, Merck/MSD, Scynexis, Vical, and received lecture honoraria from Astellas, Basilea, Gilead, Merck/MSD and Pfizer

P. Hamal has no potential conflicts of interest to declare.

H. Masoud has no potential conflicts of interest to declare.

M. Mares has no potential conflicts of interest to declare.

J.F. Meis reports grants and speaker fees from Scynexis, F2G, Gilead Sciences, United Medical, and TEVA, outside the submitted work.

S.E. Kidd has no conflict of interest to declare.

O. Kurzai has no conflict of interest to declare.

S. Oliveri has no potential conflicts of interest to declare.

A. Rodloff has no potential conflicts of interest to declare.

I. Guerrero-Lozano has no potential conflicts of interest to declare.

E. Segal has no conflict of interest to declare.

S.J. Taj-Aldeen has no conflicts of interest to declare.

A.M. Tortorano has no conflict of interest to declare.

L. Trovato has no potential conflicts of interest to declare.

G. Walther has no potential conflicts of interest to declare.

B. Willinger received a research grant and lecture honoraria from Pfizer and serves as a consultant to MSD.

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Table 1. Antifungal susceptibility patterns of *C. inconspicua*, *C. pararugosa*, *D. rugosa*, and *P. norvegensis* using EUCAST and Etest®

			EUCAST read visually			EUCAST read by reader			Etest®		
	N	Antifungal drugs	MIC_{50} (mg/L)/*range*[1]	MIC_{90} (mg/L)	Proposed ECOFF (mg/L)[2]	MIC_{50} (mg/L)/*range*[1]	MIC_{90} (mg/L)	Proposed ECOFF (mg/L)[2]	MIC_{50} (mg/L)/*range*[1]	MIC_{90} (mg/L)	**Proposed ECOFF (mg/L)[2]**
C. inconspicua	168	ITC	0.5	1.0	4.0	0.25	1.0	2.0	0.5	1.0	4.0
		POS	0.25	0.5	2.0	0.125	0.25	2.0	0.25	0.5	2.0
		ISA	0.5	1.0	4.0	0.25	0.5	2.0	0.125	0.25	1.0
		FLU	64.0	>64.0	>64.0	32.0	>64.0	>64.0	64.0	>64.0	>64.0
		VRC	0.25	0.5	2.0	0.25	1.0	2.0	0.125	0.25	1.0
		ANI	0.03	0.125	0.25	0.03	0.06	0.25	0.008	0.016	0.06
		MICA	0.06	0.125	0.5	0.06	0.125	0.5	0.03	0.06	0.25
		CAS	1.0	1.0	>4	1.0	2.0	>4.0	0.25	0.5	4.0
		AMB	0.5	1.0	4.0	0.5	1.0	>4	0.03	0.125	0.25
***D. rugosa* species complex#**	90	ITC	0.25	0.5	2.0	0.25	0.5	4.0	0.06	0.5	1.0
		POS	0.125	0.5	2.0	0.125	0.5	2.0	0.03	0.125	0.25
		ISA	0.5	1.0	4.0	0.5	1.0	4.0	0.06	0.125	0.25
		FLU	8.0	32.0	64.0	8.0	32	64.0	4.0	16.0	32.0
		VRC	0.25	1.0	2.0	0.25	1.0	2.0	0.06	0.125	0.5
		ANI	2.0	>4.0	>4.0	2.0	>4.0	>4.0	0.125	1.0	1.0
		MICA	0.25	>4.0	1.0	0.25	>4.0	>4.0	0.125	0.25	1.0
		CAS	2.0	>4.0	>4.0	2.0	>4.0	>4.0	1.0	>4.0	4.0
		AMB	0.5	1.0	>4.0	0.5	1.0	1.0	0.125	0.5	1.0
C. pararugosa	60	ITC	0.5	0.5	N/A	0.25	1.0	4.0	0.125	0.5	N/A
		POS	0.25	0.5	N/A	0.25	0.5	2.0	0.06	0.125	N/A
		ISA	0.5	1.0	N/A	0.5	2.0	4.0	0.06	0.25	N/A
		FLC	16.0	32.0	N/A	16.0	32.0	>64.0	4.0	32.0	N/A
		VRC	0.5	1.0	N/A	0.5	1.0	4.0	0.06	0.125	N/A
		ANI	1.0	>4.0	N/A	0.5	>4.0	4.0	0.125	1.0	N/A
		MICA	0.25	>4.0	N/A	0.5	>4.0	2.0	0.125	0.5	N/A
		CAS	1.0	>4.0	N/A	>4.0	>4.0	>4.0	0.5	4.0	N/A
		AMB	0.5	1.0	N/A	1.0	1.0	>4.0	0.125	0.25	N/A
D. rugosa	26	ITC	0.125	0.25	N/A	0.125	0.25	1.0	0.03	0.5	N/A
		POS	0.03	0.125	N/A	0.03	0.125	0.5	0.016	0.06	N/A
		ISA	0.03	0.125	N/A	0.016	0.06	0.125	0.016	0.03	N/A
		FLC	4.0	16.0	N/A	8.0	16.0	32.0	4.0	8.0	N/A
		VRC	0.06	0.25	N/A	0.06	0.25	0.5	0.06	0.06	N/A
		ANI	2.0	4.0	N/A	2.0	>4.0	>4.0	0.06	0.5	N/A
		MICA	0.125	>4.0	N/A	0.125	>4.0	0.5	0.125	0.25	N/A
		CAS	>4.0	>4.0	N/A	4.0	>4.0	>4.0	2.0	>4.0	N/A
		AMB	1.0	1.0	N/A	1.0	2.0	>4.0	0.5	1.0	N/A
C. mesorugosa	4	ITC	0.016-0.03	N/A	N/A	0.008-0.06	N/A	N/A	0.008	N/A	N/A
		POS	0.008-0.016	N/A	N/A	0.008	N/A	N/A	0.008	N/A	N/A
		ISA	0.008	N/A	N/A	0.008	N/A	N/A	0.008	N/A	N/A

		FLC	1.0-8	N/A	N/A	2.0-8	N/A	N/A	1.0-8	N/A	N/A
		VRC	0,008-0,016	N/A	N/A	0,008-0,03	N/A	N/A	0,008-0,016	N/A	N/A
		ANI	4.0	N/A	N/A	4.0->4.0	N/A	N/A	0,03-0,125	N/A	N/A
		MICA	>4.0	N/A	N/A	>4.0	N/A	N/A	0,06-0,125	N/A	N/A
		CAS	>4.0	N/A	N/A	2.0->4.0	N/A	N/A	2.0->4	N/A	N/A
		AMB	0.25-1.0	N/A	N/A	0.5-1.0	N/A	N/A	0.5	N/A	N/A
P. norvegensis	15	ITC	0.5	1.0	N/A	0.25	0.5	2.0	0.5	4.0	N/A
		POS	0.5	0.5	N/A	0.125	0.25	1.0	0.5	0.5	N/A
		ISA	1.0	2.0	N/A	0.5	2.0	4.0	0.25	1.0	N/A
		FLC	64.0	>64.0	N/A	64.0	>64.0	>64.0	64.0	>64.0	N/A
		VRC	1.0	4.0	N/A	1.0	2.0	>4.0	1.0	1.0	N/A
		ANI	0.03	0.125	N/A	0.03	0.125	0.25	0.016	0.016	N/A
		MICA	0.06	0.125	N/A	0.06	0.125	0.5	0.125	0.125	N/A
		CAS	0.5	1.0	N/A	0.5	1.0	4.0	0.5	0.5	N/A
		AMB	1.0	2.0	N/A	1.0	2.0	>4.00	10	1.0	N/A
C. ciferrii	8	ITC	0.5-2.0	N/A	N/A	0.5-2.0	N/A	N/A	0.25->4.0	N/A	N/A
		POS	0.25-2.0	N/A	N/A	0.125-2.0	N/A	N/A	0.5-4.0	N/A	N/A
		ISA	0.03-4.0	N/A	N/A	0.125-2.0	N/A	N/A	0.06-1.0	N/A	N/A
		FLC	32.0->64.0	N/A	N/A	16.0->64.0	N/A	N/A	64.0->64.0	N/A	N/A
		VRC	0.5-2.0	N/A	N/A	0.5-2.0	N/A	N/A	0.25-1.0	N/A	N/A
		ANI	0.25->4.0	N/A	N/A	0.03->4.0	N/A	N/A	0.008-0.125	N/A	N/A
		MICA	0.03.->4.0	N/A	N/A	0.06-0.125	N/A	N/A	0.06-0.25	N/A	N/A
		CAS	0.06->4.0	N/A	N/A	0.125->4.0	N/A	N/A	0.25	N/A	N/A
		AMB	0.5-2.0	N/A	N/A	1.0-2.0	N/A	N/A	0.5-2.0	N/A	N/A

C. (*Candida)*. *#D. rugosa* species complex (including *D. rugosa*, *C. pararugosa*, and *C. mesorugosa*), N/A (not applicable), MIC_{50} (concentration required to inhibit the growth of 50% population). MIC_{90} (concentration required to inhibit the growth of 90% population). Proposed ECOFF (ECOFF proposed in this study. third dilution step from the center of the distribution). ITC (Itraconazole). POS (Posaconazole). ISA (Isavuconazole). FLC (Fluconazole). VRC (Voriconazole). ANI (Anidulafungin). MICA (Micafungin). CAS (Caspofungin). AMB(Amphotericin B).

[1] MIC_{50} is given for species represented by ≥ 15 isolates; *range* is given for species with < 15 isolates.

[2] ECOFFs are given in bold numbers for species with ≥ 90 isolates.

Table 2. Method agreement for Etest® and EUCAST for the species *C. inconspicua*, *C. pararugosa*, *D. rugosa, and P. norvegensis*

			Agreement between EUCAST, read visually and by reader						Agreement between EUCAST and Etest●					
	N	Antifungal drugs	Essential agreement (%)						Essential agreement (%)					
			±1 Folds	±2 Folds	C.A.	Minor error	Major error	C.A ECOFF	±1 Folds	±2 Folds	C.A.	Minor error	Major error	C.A ECOFF
C. inconspicua	168	ITC	73.2	82.7	81.5			99.4	47.0	64.8	79.7			97.01
		POS	69.0	80.9	66.0			98.8	62.5	77.3	64.2			98.2
		ISA	66.0	80.9	0			96.4	60.1	80.3	N/A			96.4
		FLC	92.2	97.6	96.4	2.3	1.1	100.0	75.5	92.8	95.2	2.9	1.7	100.0
		VRC	87.5	96.4	73.8	26.1	0	97.0	60.1	83.3	42.2	52.3	5.3	98.2
		ANI	88.0	96.4	79.1			98.8	31.5	68.4	66.0			97.0
		MICA	89.2	95.2	89.2			99.4	83.3	95.8	77.3			97.6
		CAS	82.1	86.9	0			97.6	60.1	82.7	N/A			97.0
		AMB	71.4	83.9	92.8			100.0	15.4	29.7	91.6			99.4
D. rugosa species	90	ITC	88.8	97.8	89.9			100.0	33.7	65.2	58.4			95.5
complex		POS	87.6	93.3	46.4			97.8	38.2	68.5	25.6			89.9
		ISA	76.4	88.8	0			95.5	34.8	60.7	N/A			89.9
		FLC	82.0	95.5	79.8	16.9	3.4	96.6	53.9	76.4	46.1	28.1	25.8	93.3
		VRC	86.5	96.6	83.1	16.9	0	97.8	31.5	58.4	39.3	34.8	25.8	96.6
		ANI	78.7	85.4	95.5			100	21.3	39.3	71.9			92.1
		MICA	75.3	77.6	51.2			82.0	46.1	61.8	51.8			70.8
		CAS	75.3	79.8	0			100.0	49.4	62.9	N/A			89.9
		AMB	91.0	95.5	50			100.0	29.2	53.9	48.2			100
C. pararugosa	60	ITC	89.8	98.3	100.0			100.0	30.5	64.4	61.0			94.9
		POS	88.1	89.8	91.5			96.6	30.5	61.0	33.8			94.9
		ISA	67.7	84.7	0			93.2	25.4	44.0	N/A			91.5
		FLC	88.1	96.6	94.9	5.0	0	100.0	45.7	69.4	49.1	18.6	32.2	33.8
		VRC	86.4	94.9	79.6	20.3	0	94.9	18.6	42.3	16.9	44.0	38.9	96.6
		ANI	76.2	81.3	96.6			71.1	27.1	42.3	72.8			72.8
		MICA	77.9	79.6	98.3			84.7	45.7	62.7	96.6			74.5
		CAS	72.8	76.2	0			100.0	45.7	54.2	N/A			93.2
		AMB	88.1	93.2	98.3			100.0	11.8	38.9	94.9			100.0
D. rugosa	26	ITC	92.3	96.1	65.3			96.1	38.4	65.3	46.1			88.4
		POS	84.6	100.0	76.9			100.0	53.8	84.6	73.0			96.1
		ISA	92.3	96.1	0			92.3	53.8	96.1	N/A			96.1
		FLC	65.3	92.3	42.3	46.1	11.5	100.0	65.3	88.4	34.6	50.0	15.3	100
		VRC	84.6	100.0	88.4	11.5	0	100.0	53.8	88.4	80.7	19.2	0	96.1
		ANI	80.7	92.3	92.3			100.0	11.5	23.0	69.2			88.4
		MICA	65.3	69.2	92.3			73.0	53.8	69.2	100.0			65.3
		CAS	80.7	84.6	0			100.0	53.8	76.9	N/A			100.0
		AMB	96.1	100.0	84.6			100.0	69.2	92.3	80.7			100.0
P. norvegensis	15	ITC	60.0	73.3	66.6			100.0	26.6	26.6	73.3			93.3
		POS	60.0	73.3	73.3			100.0	53.3	66.6	66.6			100.0
		ISA	60.0	73.3	0			100.0	53.3	86.6	N/A			100.0
		FLC	86.6	93.3	93.3	6.6	0	100.0	93.3	100.0	100.0	0	0	100.0
		VRC	86.6	100.0	100.0	0	0	100.0	80.0	93.3	93.3	6.6	0	100.0
		ANI	80.0	93.3	66.6			100.0	33.3	66.6	53.3			100.0
		MICA	60.0	73.3	73.3			100.0	66.6	80.0	93.3			100.0
		CAS	80.0	93.3	0			100.0	66.6	93.3	N/A			100.0
		AMB	93.3	100.0	93.3			100.0	46.6	80.0	73.3			100.0

C. (*Candida*). *D. rugosa species complex* (*D. rugosa*, *C. pararugosa*, and *C. mesorugosa*), Agreement between method of the most common species in the collection. C.A. (Categorical agreement calculated

using clinical breakpoints of *Candida albicans*). C.A. ECOFF (Categorical agreement calculated using the proposed ECOFF in the study). ITC (Itraconazole). POS (Posaconazole). ISA (Isavuconazole). FLC (Fluconazole). VRC (Voriconazole). ANI (Anidulafungin). MICA (Micafungin). CAS (Caspofungin). AMB(Amphotericin B).