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Running title : Azoles distribution of 40 common & rare yeast species

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Abstract

Invasive yeast infections represent a major global public health issue and only few antifungal agents are available. Azoles are one of the classes of antifungals used for treatment of invasive candidiasis. The determination of antifungal susceptibility profiles using

standardized methods is important to identify resistant isolates and to uncover the potential emergence of intrinsically-resistant species. We here report data on 9,319 clinical isolates belonging to 40 pathogenic yeast species recovered in France over 17 years. The antifungal susceptibility profiles were all determined at the National Reference Center for Invasive Mycoses and Antifungals based on the EUCAST broth microdilution method. The centralized collection and analysis allowed us to describe the trends of azoles susceptibility of isolates belonging to common species, confirming the high susceptibility for *C. albicans* (n=3,295), *C. tropicalis* (n=641), *C. parapsilosis* (n=820), and decreased susceptibility for *C. glabrata* (n=1,274), and *P. kudriavzevii* (n=343). They also provide interesting data concerning azole susceptibility of *Cr. neoformans* species complex: showing comparable MICs distribution for the three species but lower MIC50 and MIC90 for serotype D (n=208) compared to serotype A (n=949) and AD hybrids (n=177). Finally, these data provide useful information for rare and/or emerging species such as *C. lusitaniae* (n=221), *S. clavata* (n=184), *M. guilliermondii* complex (n=150), *C. haemulonii* complex (n=87), *R. mucilaginosa* (n=55), *W. anomalus* (n=36).

Introduction

Invasive fungal infections (IFIs) represent a major worldwide public health issue with an incidence of 5.9 to 20.3 cases/100 000 patients per year in France, up to 27.2 / 100 000 in USA and 14.1/100 000 in the United Kingdom (1-5). Despite treatment, the mortality remains high, ranging from 7.5 to 27.6%, with an estimation of 1.5 million deaths annually (6). Yeasts are the main causative agents of these infections and only few antifungals are effective against the most common Ascomycetous yeasts, and even less against Basidiomycetous yeasts. The azoles are the largest class of antifungal agents used for treatment of IFIs, especially fluconazole for treatment of candidemia (7, 8). Azoles inhibit the 14-alpha-lanosterol

demethylase (Cyp51), resulting in depletion of ergosterol synthesis, a major component of the fungal cell wall and in accumulation of toxic 14-alpha-demethylated sterols in the membrane (9). The determination of *in vitro* antifungal susceptibility allows determination of wild type population and those with acquired or mutational resistance to the drug. Therefore, epidemiological surveys and standardized methods of antifungal susceptibility testing are important to confirm or not that the antifungal susceptibility of a given species remains unchanged/stable. Among internationally recognized standards, two broth microdilution methods are well standardized by the EUCAST (European Committee on Antimicrobial Susceptibility Testing) and the CLSI (Clinical and Laboratory Standards Institute) for determining minimal inhibitory concentrations (MICs). Expert committees in both instances establish breakpoints (BPs) and epidemiological cut-off (abbreviated ECOFFs for EUCAST, and ECVs for CLSI) values in order to easily distinguish between susceptible and resistant and wild-type and non-wild-type isolates, respectively. BPs are defined for a limited number of species and antifungal agents based on MICs distributions, clinical data, pharmacodynamics and pharmacokinetics of the drugs while ECOFFs are determined based only on MICs distributions. An ECOFF corresponds to the MIC that separates a population of isolates into wild-type and non-wild type. Unlike BP, ECOFF does not necessarily predict clinical failure but it can be helpful for clinical decision (SOP10.1 https://eucast.org/documents/sops/ (10)). The EUCAST Antifungal Susceptibility Testing subcommittee (EUCAST-AFST) regularly updates data concerning MICs distributions, and ECOFFs for frequent species involved in human infections based on data generated by more than 15 European centers (http://www.eucast.org/astoffungi/clinicalbreakpointsforantifungals/, (11)). Furthermore, all over the world, mono- or multicentric studies provide reports adding to the knowledge on antifungal susceptibility profiles, mostly of common yeast species (12-20). For rare species

however, obtaining robust data is difficult given the low number of reported cases (12). Centralized data are therefore important for these species to determine their normal susceptibility profile (10).

The French National Reference Center for Invasive Fungal Infections and Antifungals (NRCMA) provides antifungal susceptibility testing results using the EUCAST method for all the isolates collected through its missions of expertise on pathogenic fungi and surveillance of IFIs in France. We here report the results obtained for azoles on more than 9,000 clinical isolates belonging to 40 different species, collected nationwide in France between 2003 and 2019. These data provide an overview of the susceptibility to azoles of frequent and rare pathogenic yeast species in France.

Results

A total of 9,319 yeast isolates were included in the analysis. These isolates, received at the NRCMA between the 1st of September 2003 and the 31st December of 2019, were mainly involved in IFIs (80.8%; 6,853 recovered from blood cultures, and 679 from cerebrospinal fluids or brain abscesses cultures). The isolates belonged to 40 different species (32/40 were Ascomycetes corresponding to 17 genera, and 8/40 were Basidiomycetes belonging to 4 genera). Eleven species were represented by more than 100 isolates: *Candida albicans, Candida glabrata, Candida tropicalis, Candida parapsilosis* and *Pichia kudriavzevii* (syn. *Candida krusei*) and *Cryptococcus neoformans* complex followed by *Clavispora lusitaniae, Kluyveromyces marxianus, Saprochaete clavata*, *Candida dubliniensis* and *Meyerozyma guilliermondii* (Table 1). Ranges of MICs, MIC50 and MIC90 for fluconazole, voriconazole and posaconazole are listed in Table 1 while MICs distributions are presented in Table S1. Of note, while the median MIC values for each batch of QC strains (ATCC22019 and ATCC6258) were always in the range of acceptable MIC defined by EUCAST-AFST for

fluconazole and voriconazole, they were one dilution higher for posaconazole between 2011 and 2015. However, the proportion of posaconazole-resistant (MIC>0.06mg/L) isolates was not higher at that time (Figure 1).

We first compared the MIC distributions recorded at the NRCMA to those available in the EUCAST dataset. For fluconazole and voriconazole, ranges of MICs were similar or with a maximum variation of 2 dilutions. Medians of MICs were equal or with a maximum of one dilution difference for *C. albicans, C. dubliniensis, C. glabrata, C parapsilosis, C. tropicalis, P. kudriavzevii, W. anomalus, K. marxianus, C. lusitaniae* and *M. guilliermondii* (data not shown). For posaconazole, MIC distributions were also similar except for *C. parapsilosis, C. tropicalis* and *M. guilliermondii* (Supplemental Figure 1) with a median MIC value for the NRCMA dataset 2 dilutions higher than for the EUCAST dataset.

Among the *Candida parapsilosis* complex, the three species had similar MIC distribution for posaconazole while *C. metapsilosis* isolates had lower MICs for voriconazole and fluconazole but a higher MIC50 for fluconazole than *C. parapsilosis* and *C. orthopsilosis*. Using EUCAST BP for fluconazole (R>4mg/L), *C. orthopsilosis* has the highest number of resistant isolates (12.2%) (Table 1, Figure 2).

For *Cr. neoformans*, range of MICs, MIC50 and MIC90 were determined for serotype A, serotype D and AD hybrids. The distribution of MICs was comparable for the three species but for the three azoles, serotype D isolates exhibited lower MIC50 and MIC90 than serotypes A and AD hybrids (Table1 and Table S1).

Using the EUCAST breakpoints (BP) for fluconazole, 32.4% *C. glabrata* isolates were resistant (R > 16mg/L) compared to less than 7.5% of the isolates for all the other common species (R>4 mg/L) (Table 1). Using the non-species related BP defined by EUCAST for fluconazole (R>4mg/L), four species (*C. haemulonii, C. duobushaemulonii, C.*

palmioleophila, C. auris) had more than 90% of resistant isolates while three others (*C. metapsilosis*, *C. orthopsilosis* and *C. nivariensis*) had 2.2%, 12.2% and 7.7% of fluconazole-resistant isolates, respectively. According to the BPs for posaconazole (R>0.25 mg/L) and voriconazole (R>0.06 mg/L), the percentage of resistant isolates was 23.9% and 2.2% for *C. parapsilosis* and 27.3% and 9.5% for *C. tropicalis*, respectively (Table 1).

According to the EUCAST ECOFF, 26.8% of *P. kudriavzevii* (ECOFF=128mg/L) and 20% of *M. guilliermondii* (ECOFF=16mg/L) isolates were considered as non-wild type for fluconazole. Likewise, 9.6% of *C. glabrata* (ECOFF=1mg/L), 4.5% of *C. lusitaniae* (ECOFF=0.06mg/L), 13.9% of *M. guilliermondii* (ECOFF=0.25mg/L) and 2.6% of *P. kudriavzevii* (ECOFF=1mg/L) isolates were considered as non-wild type for voriconazole (Table 1).

Among the population of *C. glabrata* isolates, 7.9 % (101/1274) were simultaneously resistant to fluconazole and considered non-wild-type for voriconazole. Among the 155 *C. albicans* isolates resistant to at least one azole, 49 (31.6%) were resistant to the three azoles. Cross-resistance to the three azoles concerned 0/8 *C. dubliniensis*, 29/192 (15.1%) *C. tropicalis*, and 7/236 (3.0%) *C. parapsilosis* (Supplemental Figure 2). The majority (>80%) of isolates resistant to at least one azole were resistant to posaconazole. The proportion of posaconazole-resistant isolates varied according to the year of isolation for *C. tropicalis* and *C. parapsilosis* (Figure 1). This variation was correlated with variation of voriconazole-resistance for *C. tropicalis.*

Discussion

In the present study, we report the azoles susceptibility profiles for 40 yeast species involved in IFIs based on the MIC distribution of 9,319 clinical isolates recovered in France between 2003 and 2019. We provide azole profiles for common, emerging and rare species thanks to

our varied and important collection of isolates. Our results confirm that *C. albicans, C. dubliniensis, C. tropicalis, C. parapsilosis* complex, *K. marxianus* and *C. lusitaniae* can be considered as susceptible *in vitro* to fluconazole, voriconazole and posaconazole (14, 16, 18, 19, 21), while (i) *C. neoformans* complex has decreased *in vitro* susceptibility to fluconazole, (ii) *P. kudriavzevii* and uncommon species *C. haemulonii* complex, *M. guilliermondii* complex, *P. norvegensis, S. clavata, G. candidus* and *R. mucilaginosa* can be considered as intrinsically resistant to fluconazole (MIC90≥ 64mg/L) (17, 19, 20, 22-24). Of note, *C. haemulonii* and *C. duobushaemulonii*, which belong to the same species complex and are closely related to the emerging *C. auris,* can also be considered intrinsically resistant to voriconazole and posaconazole (19, 25, 26). Other uncommon species such as *S. cerevisiae, K. ohmeri, C. palmioleophila, P. cactophila, M. capitatus, Y. lipolityca, Cr. gattii* and *T. asahii* should be considered as less susceptible *in vitro* to fluconazole, with "intermediate" MIC value (MIC90≥16mg/L) (27, 28) while voriconazole seems to be the most potent azole *in vitro,* which is already known for *Trichosporon* spp. (7, 23). When considering the *C. parapsilosis* complex, *C. metapsilosis* was the species the most sensitive *in vitro* to azoles with the lowest percentage of fluconazole-resistant isolates while *C. orthopsilosis* seemed to be more resistant than the other species of the complex with the highest percentage of fluconazole-resistant isolates and the highest MIC90 for fluconazole, voriconazole and posaconazole (12, 19). There may be a link between the low frequency of *C. metapsilosis* recovered from IFIs in the literature (29) and this highest azole sensitivity. Another hypothesis proposed by Gago *et al.* is that *C. metapsilosis* has a reduced ability to produce some of the known virulence factors (30). Finally, we confirmed that very rare species *L. elongisporus* and *Cy. fabianii* were susceptible to the three azoles while *W. anomalus, K. ohmeri* and *Cy. jadinii* were less susceptible to fluconazole with "intermediate" MICs (12, 31-38).

Despite reports of azole resistance acquired after treatment, in common and rare yeast species, and description of an increasing percentage of resistant isolates (39), this phenomenon remains rare (1, 16, 40, 41) or has geographical specificity (42, 43), and seems to concern especially *C. tropicalis*, *C. glabrata* and *C. parapsilosis* (17). In the present study, we observed a proportion of fluconazole-resistant isolates among *C. albicans* and *C. parapsilosis* isolates similar to that reported in international surveys (17, 19, 20, 28, 44). We observed posaconazole-resistant isolates of *C. tropicalis* and *C. parapsilosis* complex (23.9 and 27.3%, respectively), with a variable proportion, according to the year of isolation. The increased proportion of posaconazole-resistant *C. tropicalis* isolates correlated with an increased proportion of voriconazole-resistance of those isolates. This heterogeneous percentage of resistant isolates in our collection remained unexplained. Indeed, we raised a few hypotheses: (i) Technical issues related to the batch of antifungal powder or RPMI medium that translated into a higher median posaconazole and not the other azoles MIC for the QC strains, but this was not associated with an increase in the proportion of posaconazole-resistant isolates in the related series; (ii) Geographic specificity and/or local outbreak could be responsible as described for instance in the multicentric CHIF-Net study (19) and in a national survey in Belgium (43), but none was reported to our knowledge; (iii) Bias related to the isolates analyzed knowing that 22% of the isolates were sent for expertise following therapeutic failure and not part of the epidemiological surveys (2, 45). However, posaconazole-resistance was not restricted to the isolates sent for expertise.

We also report an important percentage of *C. glabrata* isolates resistant to fluconazole (32.4%). The proportion of *C. glabrata* resistant to fluconazole seems to be very heterogeneous according to the country, the center and the year of isolation. In fact, Xiao *et al*. reported 10.3 to 19% of resistant isolates in China between 2009-2017 (19, 20, 23), based on the CHIF-Net study, while Pfaller *et al*. published 0 to 28.6%, according to the year of

isolation in the SENTRY study (44), Bassetti *et al.*, reported a percentage of 0 to 21.2% in the SENTRY and ARTEMIS studies depending on the continent (46), Borman *et al.* and Trouvé *et al*. reported 12.7% and 11.3% of resistance, respectively in national studies (28, 43). Of note, the BP values for *C. glabrata* was modified by EUCAST-AFST committee in 2020, from R>32mg/L to R>16mg/L (11). When using the previous BP (MIC>32mg/L), 18.8% of *C. glabrata* in our collection was identified as resistant to fluconazole, which is comparable to the studies published earlier before the change of the BP threshold.

Our data confirm azole susceptibility profiles for frequent species and, to our knowledge, it is one of the only studies to assemble MIC data for more than 40 pathogenic yeast species, mainly involved in invasive infection, including big samples of isolates, even for rare and emerging species such as *C. lusitaniae* (n=221), *S. clavata* (n=184), *M. guilliermondii* complex (n=150), *C. haemulonii* complex (n=87), *R. mucilaginosa* (n=55), *W. anomalus* (n=36) (12). This emphasizes the importance of centralization of isolates for collection and analysis by National Reference Centers. Our results confirm that an antifungal susceptibility profile is associated with each species, hence the importance of accurate yeast species identification as soon as possible to infer the susceptibility profile. Of course, it goes without saying that the determination of MICs, using standardized methods, remains important for rare species and the monitoring of potential emergence of resistance including in the context of therapeutic failure.

Materials & Methods

Isolates

Between the 1st January of 2003 and the 31st December of 2019, a total of 9,449 yeast were sent to the National Reference Center for Mycosis & Antifungal agents (NRCMA). We

analyzed MICs data for 9,319 isolates belonging to the species for which at least 5 isolates were available. Majority of the isolates were sent for expertise (7358/9,319; 78.8%; *i.e.* species identification, antifungal susceptibility testing) and/or as participation to epidemiological surveys. For rare species represented by 10 or less isolates, all isolates were recovered from different patients.

Species identification

For all isolates, purity was checked on chromogenic medium (BBL™ Chromagar™ Candida Medium, BD, GmbH) or Niger seed agar for *Cryptococcus* spp.. Phenotypic identification was performed using carbon assimilation profiles (ID32C, BioMérieux, Marcy-l'Etoile, France) before 2014, and matrix assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF, MALDI Biotyper, Bruker Daltonik, GmbH) since 2014. Duplex PCR was performed to differentiate *Candida dubliniensis* and *Candida albicans* (47). For all isolates, PCR and sequencing of ITS1-5.8S-ITS2 and D1D2 regions were performed, except for *C. glabrata*, *C. tropicalis*, *P.kudriavzevii* and *K. marxianus*, using V9D/LS266 (48, 49) and NL1/NL4 (50) primers, respectively. In addition, part of the actin gene (for *C. lusitaniae* (51) and *Debaryomyces* species (52)), part of the RPBI gene (for *M. guilliermondii*, *M. caribbica*, *C. carpophila* (53)) or the IGS1 region (for *Trichosporon* species (54)) were sequenced. Sequences were compared to sequences of the type strain of each species and of the closely related species. Serotype was determined for *Cr. neoformans* isolates by PCR amplification of part of GPA1 and PAK1 genes using a triplex PCR with primers specific of the serotype previously described (55).

Antifungal susceptibility

Minimal inhibitory concentrations (MICs) were determined for all isolates, for 3 antifungal agents (fluconazole provided by Pfizer Inc (NewYork, USA), Sigma-Aldrich (Merck KGaA,

Darmstadt, Germany) and Alsachim (Shimadzu Group Company, France); voriconazole provided by Pfizer and Alsachim and posaconazole provided by SheringPlough (Merck & Co. Inc., USA) and Alsachim) by using the standardized broth microdilution method EUCAST following the procedure E. DEF 7.3.2 (https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Files/EUCAST_E_Def_7.3.2_Yeast_testing_definitive_revised_2020.pdf), in 96 plate (sterile Tissue culture plates, 96 well flat bottom in clear polystyrene, TPP® Techno Plastic Products AG, Switzerland, Reference 92096). The concentrations tested ranged between 0.0015 mg/L to 8 mg/L for posaconazole and voriconazole and between 0.125 mg/L to 64 mg/L for fluconazole. QC strains (ATCC22019, ATCC6258) were included in each set. The concentrations corresponding to the MIC that inhibited 50% (MIC50) and 90% (MIC90) of the isolates were determined for species having 10 or more isolates.

Our dataset (MIC distribution) was compared with that available on the EUCAST website (https://mic.eucast.org/Eucast2/SearchController/search.jsp?action=init, June 2020).

The BP or ECOFF values determined by EUCAST for some species and some antifungal agents (https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Clinical_breakpoints/AFST_BP_v10.0_200204.pdf) were used to calculate percentage of resistant (R) isolates and non-wild-type (NWT), respectively. Non-species related BP for fluconazole, defined by EUCAST, (MIC >16 mg/L) for *Candida* were also used to calculate percentage of resistant isolate for *C. orthopsilosis, C. metapsilosis, C. nivariensis, C. haemulonii, C. duobushaemulonii* and *C. palmioleophila*. Since not all isolates were collected through unbiased epidemiological survey (7358/9,319; 78.8%), we did not determine local ECOFF, and reported only the percentage of resistant or non-wild-type isolates in our collection.

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Figure Legends

Figure 1. Percentage of fluconazole, voriconazole and posaconazole -resistant isolates, according to the year of isolation, for (A) *Candida albicans.* The proportion of resistant isolates being similar for posaconazole and fluconazole between 2016 and 2019, the lines are

overlaid***;*** **(B)** ***Candida parapsilosis,*** **and (C)** ***Candida tropicalis*** (voriconazole and fluconazole lines overlaid between 2015 and 2019).

Figure 2. MIC distribution of *Candida parapsilosis* complex isolates for (A) Voriconazole, (B) Fluconazole and (C) Posaconazole. EUCAST breakpoint values (R) for *C. parapsilosis sensu stricto* are indicated in the graphs.

Supplemental Figure 1. Comparison of EUCAST and CNRMA data for posaconazole MIC distribution of *C. tropicalis* (A), *C. parapsilosis* (B) and *M. guilliermondii*. EUCAST breakpoint value (R) or ECOFF value are indicated in the graphs.

Supplemental Figure 2. Azoles cross-resistance. Graphs represent the number of isolates resistant for each azoles, for two or three azoles among isolates resistant for at least one azoles for **(A)** ***Candida albicans,*** **(B)** ***Candida dubliniensis,*** **(C)** ***Candida tropicalis*** **and (D)** ***Candida parapsilosis***

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