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1 **Method-dependent epidemiological cutoff values (ECVs) for detection of triazole**
2 **resistance in *Candida* and *Aspergillus* species for the SYO colorimetric broth and**
3 **Etest agar diffusion methods**
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9 A. Espinel-Ingroff[1*], J. Turnidge[2], A. Alastruey-Izquierdo[3], F. Botterel[4], E. Canton[5], C. Castro[6], Y-
10 C Chen[7]; Y Chen[8], E. Chryssanthou[9], E. Dannaoui[10], G. Garcia-Effron[11], G. M. Gonzalez[12], N. P.
11 Govender[13], J. Guinea[14], S. Kidd[15], M. Lackner[16], C. Lass-Flörl[16], M.J. Linares-Sicilia[17], L.
12 López-Soria[18],R. Magobo[13], T. Pelaez[19], G. Quindós[20], M. A. Rodriguez-Iglesia[21], M. A. Ruiz[22], F.
13 Sánchez-Reus[23], M. Sanguinetti[24], R. Shields[25], P. Szweda[26], A. Tortorano[27], N. L. Wengenack[28],
14 S. Bramati[29], C. Cavanna[30], C. DeLuca[31], M. Gelmi[32], A. Grancini[33], G. Lombardi[34], J.
15 Meletiadis[35], C. E. Negri[36], M. Passera[37], J. Peman[38], A. Prigitano[27], E. Sala[39], M. Tejada[40]
16
17 [*1]VCU Medical Center, Richmond, VA, USA; [2]University of Adelaide, Adelaide, Australia;
18 [3]Mycology Reference Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III,
19 Majadahonda, Madrid, Spain; [4]Unité de Parasitologie, Mycologie, Département de Bactériologie
20 Virologie Hygiène Mycologie Parasitologie, Créteil, France; [5]Grupo de Infección Grave, Instituto
21 de Investigación Sanitaria La Fe, Valencia, Spain; [6]Unidad de Gestión Clínica de Enfermedades
22 Infecciosas y Microbiología, Hospital de Valme, Seville, Spain; [7]Department of Internal
23 Medicine, National Taiwan University Hospital and College of Medicine, Taipei, Taiwan; [8]Public
24 Health Ontario, Toronto, Ontario, Canada; [9]Klinisk Mikrobiologi, Karolinska,
25 Universitetlaboratoriet, Karolinska, Universitetssjukhuset, Stockholm, Sweden; [10]Université
26 Paris-Descartes, Faculté de Médecine, APHP, Hôpital Européen Georges Pompidou, Unité de
27 Parasitologie-Mycologie, Service de Microbiologie, Paris, France; [11]Laboratorio de Micología y
28 Diagnóstico Molecular, Cátedra de Parasitología y Micología, Facultad de Bioquímica y
29 Ciencias Biológicas, Universidad Nacional del Litoral, Consejo Nacional de Investigaciones
30 Científicas y Tecnológicas (CONICET), Santa Fe, Argentina; [12]Universidad Autónoma de Nuevo
31 León, Mexico; [13]National Institute for Communicable Diseases (Centre for Healthcare-
32 Associated Infections, Antimicrobial Resistance and Mycoses), a Division of the National Health
33 Laboratory Service and Faculty of Health Sciences, University of the Witwatersrand,
34 Johannesburg, South Africa; [14]Hospital General Universitario Gregorio Marañón, Madrid, Spain;
35 [15]SA Pathology, National Mycology Reference Centre, Adelaide, Australia; [16]Division of Hygiene

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36 and Medical Microbiology, Medical University of Innsbruck, Innsbruck, Austria; [17]Departamento
37 de Microbiología, Facultad de Medicina y Enfermería, Universidad de Córdoba, Córdoba, Spain;
38 [18]Hospital Universitario Cruces, Barakaldo, Spain; [19]Hospital Universitario Central de Asturias,
39 (HUCA), Fundación para la Investigación Biomédica y la Innovación Biosanitaria del Principado
40 de Asturias (FINBA). Asturias, Spain; [20]Universidad del País Vasco/Euskal Herriko
41 Unibertsitatea, UPV/EHU, Bilbao, Spain; [21]Departamento de Biomedicina, Biotecnología y
42 Salud Pública, Universidad de Cádiz, Cadiz, Spain; [22]IIS Instituto de Investigación Sanitaria
43 Aragón, Zaragoza, Spain; [23]Hospital de la Santa Creu i Sant Pau, Barcelona, Spain; [24]Institute
44 of Microbiology, Università Cattolica del Sacro Cuore, Rome, Italy; [25]University of Pittsburgh,
45 Pittsburgh, Pennsylvania, USA; [26]Department of Pharmaceutical Technology and Biochemistry,
46 Faculty of Chemistry, Gdańsk University of Technology, Gdańsk, Poland; [27]Department of
47 Biomedical Sciences for Health, Università degli Studi di Milano, Milan, Italy; [28]Mayo Clinic,
48 Rochester, MN, USA; [29]Microbiology Laboratory, Ospedale San Gerardo, Monza, Italy;
49 [30]Microbiology and Virology Unit IRCCS Policlinico San Matteo, Pavia, Italy; [31]Microbiology
50 Section, Humanitas Research Hospital, Milan, Italy; [32]Microbiology Laboratory, A.O. Spedali
51 Civili, Brescia, Italy; [33]Microbiology Laboratory, Fondazione IRCCS Cà Granda O. Maggiore
52 Policlinico, Milan, Italy; [34]Microbiology Laboratory, Niguarda Hospital, Milan, Italy; [35]Clinical
53 Microbiology Laboratory, Attikon Hospital, Medical School, National and Kapodistrian, University
54 of Athens, Athens, Greece; [36]Laboratório Especial de Micologia, Disciplina de Infectologia,
55 Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, SP, Brazil;
56 [37]Microbiology Institute, ASST 'Papa Giovanni XXIII', Bergamo, Italy; [38]Unidad de Micología,
57 Servicio de Microbiología, Hospital Universitario La Fe, Valencia, Spain; [39]Microbiology-ASST
58 Lariana, Como, Italy; and [40]Medicina di Laboratorio, IRCCS Policlinico San Donato, Milan, Italy.
59
60 *Corresponding author address: 3804 Dover Rd., Richmond, VA 23221
61 Email: victoria.ingroff@vcuhealth.org
62
63 **Running title**: SYO and Etest Triazole ECVs for *Aspergillus* and *Candida*
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70 **Abstract**

71

72 Although the Sensitrite Yeast-One (SYO) and Etest methods are widely utilized,
73 interpretive criteria are not available for triazole susceptibility testing of *Candida* or *Aspergillus*
74 species. We collected fluconazole, itraconazole, posaconazole and voriconazole SYO and Etest
75 MICs from 39 laboratories representing all continents for (method-agent-dependent): 11,171
76 *Candida albicans*, 215 *C. dubliniensis*, 4,418 *C. glabrata* species complex (SC), 157
77 *C.* (*Meyerozyma*) *guilliermondii*, 676 *C. krusei* (*Pichia kudriavzevii*), 298 *C.* (*Clavispora*)
78 *lusitaniae*, 911 and 3,691 *C. parapsilosis sensu stricto* (*SS*) and *C. parapsilosis SC*,
79 respectively, 36 *C. metapsilosis*, 110 *C. orthopsilosis,* 1,854 *C. tropicalis*, 244 *Saccharomyces*
80 *cerevisiae*, 1,409 *Aspergillus fumigatus*, 389 *A. flavus*, 130 *A. nidulans*, 233 *A. niger*, and 302
81 *A. terreus* complexes. SYO/Etest MICs for 282 confirmed non-WT isolates were included:
82 *ERG11* (*C. albicans*), *ERG11* and *MRR1* (*C. parapsilosis*), *cyp51A* (*A. fumigatus*), and *CDR2*,
83 *CDR*1 overexpression (*C. albicans* and *C. glabrata*, respectively). Interlaboratory modal
84 agreement was superior by SYO for yeast spp., and by the Etest for *Aspergillus* spp.
85 Distributions fulfilling CLSI criteria for ECV definition were pooled and we proposed SYO ECVs
86 for *S. cerevisiae*, 9 yeast and 3 *Aspergillus* species, and Etest ECVs for 5 yeast and 4
87 *Aspergillus* species. The posaconazole SYO ECV of 0.06 μg/ml for *C. albicans* and the Etest
88 itraconazole ECV of 2 μg/ml for *A. fumigatus* were the best predictors of non-WT isolates.
89 These findings support the need for method-dependent ECVs, as overall, the SYO appears to
90 perform better for susceptibility testing of yeast spp. and the Etest for *Aspergillus* spp. Further
91 evaluations should be conducted with more *Candida* mutants.

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104 **Introduction**

105

106 The triazoles (fluconazole, isavuconazole, itraconazole, posaconazole, and
107 voriconazole) are the current treatments for severe candidiasis and aspergillosis (e.g., first-line
108 or prophylactic, adjunctive, empirical, transition from another agent, salvage therapies) (1-3).
109 These fungal infections may cause elevated levels of morbidity and mortality among
110 immunocompromised patients (3-5). The impact of azole resistance and its prevalence has
111 been widely recognized and various mechanisms of mutational resistance have been elucidated
112 in the four most common species of *Candida*, especially in *Candida albicans*, and in *Aspergillus*
113 *fumigatus* (6-10). In most *Candida* isolates, azole resistance (or unusually high or increased
114 MICs) are mostly associated with two main molecular mechanisms among others: an increase
115 (overexpression) of the azole target azole sterol demethylase or alterations (amino acid
116 substitutions) in either the gene *ERG11* as the enzyme is encoded during the fungal ergosterol
117 biosynthesis pathway or the *MRR1* transcriptional regulator (6,8,9). However, in the case of *C.*
118 *glabrata*, azole resistance has been frequently related to the overexpression or alteration of the
119 *PDR1* gene that regulates efflux pumps (7). On the other hand, the main azole resistance
120 mechanism in *A. fumigatus* is due to alterations of the *cyp51A* gene (10).

121

122 Azole susceptibility testing (yielding minimal inhibitory concentrations [MICs]) is
123 recommended for all bloodstream and other clinically relevant *Candida* isolates (1). Although
124 routine MIC determination for *Aspergillus* spp. isolates is not usually recommended during initial
125 aspergillosis therapy, MICs have an important role in identifying potentially resistant isolates,
126 e.g., isolates from patients failing therapy (2). There are several antifungal susceptibility
127 methods for the determination of MICs for isolates of both *Candida* and *Aspergillus*, including
128 the broth microdilution M27 and M38 reference methods by the Clinical and Laboratory
129 Standards Institute (CLSI) (11,12) and the Antifungal Subcommittee of the European Committee
130 on Antimicrobial Susceptibility Testing (EUCAST) (13) (http://www.eucast.org/ast_of_fungi/). In
131 addition, the colorimetric broth microdilution Sensititre Yeast-One (SYO; Trek Diagnostic
132 System, Cleveland, [OH]) as well as the agar diffusion Etest (bioMérieux, Marcy l'Etoile,
133 France), among other commercial assays, are widely utilized for antifungal susceptibility testing
134 in the clinical laboratory; these methods are more practical and less time-consuming for routine
135 use (14-16).

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137 The objective of earlier studies evaluating the performance of the SYO and Etest
138 methods involved the comparison of azole MICs obtained by these methods with those obtained
139 by the reference assays for prevalent species of *Candida* and *Aspergillus* (17-19). Some of
140 those early studies also evaluated the agreement on the ranking of isolates within existent
141 categorical endpoints with little attention to the critical issue of interlaboratory reproducibility.
142 Recently, triazole MIC data for *A. fumigatus* and *C. glabrata* mutant strains have been reported
143 by these commercial methods (20-24). However, lack of suitable clinical data has precluded the
144 establishment of breakpoints (BPs) for the categorical interpretation of triazole MICs for either
145 *Candida* or *Aspergillus* spp. by these two methods. Therefore, both assays rely on CLSI
146 available BPs for *Candida* spp. as interpretive categories as well as for quality control (QC)
147 (14,16). The proposal of SYO/Etest ECVs (epidemiological cutoff values) for susceptibility
148 testing of either *Candida* or *Aspergillus* isolates with amphotericin B or the echinocandins has
149 revealed substantial method-dependent differences between some of those values despite the
150 regulatory requirement to show equivalence to the reference method before marketing (25,26).
151 Those results emphasize the need to establish method-dependent triazole ECVs for these two
152 widely used commercial methods for testing the susceptibility of *Candida* and *Aspergillus*
153 isolates to the triazoles in the clinical laboratory.

154 For the last two years, we have gathered available triazole MICs by both SYO or Etest
155 assays for isolates of prevalent and non-prevalent yeast species (*C. albicans*, *C. dubliniensis*,
156 *C. glabrata* species complex, [SC], *C.* [*Meyerozyma*] *guilliermondii*, *C. krusei* [*Pichia*
157 *kudriavzevii*], *C.* [*Clavispora*] *lusitaniae*, *C. parapsilosis* SC, including *C. parapsilosis sensu*
158 *stricto* [*SS*], *C. orthopsilosis* and *C. tropicalis*), *Saccharomyces cerevisiae* and five *Aspergillus*
159 species complexes (*A. fumigatus* [including *A. fumigatus SS*], *A. flavus*, *A. nidulans*, *A. niger*,
160 and *A. terreus*). Additional SYO MIC distributions for less prevalent or common yeast species *C.*
161 *famata* (*Debaryomyces hansenii*), *C. kefyr* (*Kluyveromyces marxianus*), and *C. metapsilosis*
162 also were reported when they originated from at least three laboratories and had comparable
163 modes. From here on we will be using the most "common" clinical names. These triazole MICs
164 were submitted from 39 independent worldwide laboratories (method/agent/species dependent)
165 in order: (i) to define MIC distributions by each commercial susceptibility testing method/agent
166 and species; (ii) to examine the suitability of these distributions for ECV setting, including the
167 evaluation of interlaboratory modal agreement; and (iii) to define ECVs for each
168 species/agent/method that fulfilled the CLSI criteria for ECV definition (modal compatibility
169 among the laboratories, at least 100 MICs for each species/method/agent that originated in ≥3

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170 independent laboratories) using the iterative statistical method at the 97.5% cutoff value (27-29)
171 or the second numerical derivative method when the putative wild-type mode was at the lowest
172 concentration in the distribution (30).

173 Although the majority of the isolates evaluated were not assessed for mechanisms of
174 resistance, we also collected MIC data for 282 known or confirmed mutants (non wild-type [non-
175 WT]) by both methods as follows: SYO and Etest MICs for *C. albicans* (*ERG11*), SYO MICs for
176 *C. parapsilosis* (*ERG11*, *MRR1*) mutants and/or strains with overexpression of the *CDR2* gene,
177 *C. glabrata* MICs with overexpression of the *CDR1* gene, and SYO and Etest MICs for *A.*
178 *fumigatus* SS harboring *cyp51A* mutations. These data were submitted mostly from European
179 laboratories as well as from Argentina, Thailand, South Africa and one published Etest study
180 (20). SYO data for 58 *PDR* gene *C. glabrata* mutants also were submitted, but those data were
181 not included due to large modal variability of the non-mutants as compared with the global
182 modes.
183
184 **Results and Discussion**
185
186 Antimicrobial susceptibility testing for clinical isolates is most useful when either method-
187 and species-dependent BPs or ECVs are available for the isolate and agent evaluated. The BP
188 categorizes the isolate as either susceptible or resistant and the ECV as either wild-type (WT,
189 no detectable phenotypic resistance) or non-WT (more likely harboring resistance mechanisms)
190 (27). Since ECVs are based solely on in vitro data (either MIC or MEC results), classification of
191 an isolate as a presumptively WT cannot directly predict a successful therapeutic outcome.
192 Classification of an isolate as a non-WT indicates that it could harbor acquired resistance
193 mechanisms to the agent being evaluated and would less likely respond to contemporary
194 therapy (27). However, the putative mechanism of resistance would not necessarily be known in
195 order to categorize a strain as non-WT. CLSI BPs are based on in vitro and clinical data, genetic
196 mechanisms of resistance as well as pharmacokinetic/pharmacodynamics parameters (27,28).
197 EUCAST ECVs and BPs are based on MIC distributions and PK/PD parameters
198 (http://www.eucast.org/ast_of_fungi/). Therefore, when the BP is available for the isolate and
199 agent being evaluated that is the value that should be used. To our knowledge, method-
200 dependent SYO or Etest ECVs or BPs for the four triazoles evaluated have not been proposed
201 for categorization of *Candida* or *Aspergillus* isolates. Our ECVs were defined following the
202 criteria recently published by the CLSI (27). They were based on either SYO or Etest triazole
203 MIC distributions that originated from 3 to 30 (SYO) or 3 to 11 (Etest) laboratories (species and

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204 agent dependent) (Tables 1-4) (27). As mentioned before, SYO MICs were submitted from
205 multiple laboratories for the following mutants: 59 *C. albicans ERG11* (4 laboratories) and 39 *A.*
206 *fumigatus* SS *cyp51A* (5 laboratories), Etest MICs for 81 *A. fumigatus cyp51A* (7 laboratories
207 and one published study) (20) (Tables 1, 2 and 5). SYO MICs were received from single
208 laboratories for the following mutants: 13 *C. glabrata* and 2 *C. albicans* with overexpression of
209 the *CDR1* and *CDR2* genes, respectively, 78 *C. parapsilosis* (49 *ERG11* and 29 *MRR1*,
210 respectively); and Etest MICs for 10 *C. albicans* (*ERG11*) (not listed in Tables 1, 2, or 5). The
211 MICs for these confirmed mutants provided a preliminary assessment of the utility of our
212 proposed ECVs in recognizing the non-WT strains. Therefore, since BPs are not available for
213 these commercial methods, the proposed ECVs in the present study could help the clinician and
214 laboratory personnel in identifying isolates with possible acquired resistance mechanisms or
215 could be useful for surveillance or epidemiological studies.
216
217 Although SYO MICs for the species evaluated originated from 30 of the 39 participant
218 centers, exclusions were made according to the CLSI criteria for ECV definition (Table 1) (27).
219 During data consolidation, individual SYO MIC distributions of *Candida* and *Aspergillus* were not
220 included in the ECV analysis due to: aberrant or not defined modes, bimodal, or when the
221 particular mode for a distribution was more than 1 to 2 dilutions from the global mode, or when
222 there were less than five isolates in the distribution. MIC distributions were also excluded when
223 the MIC data for the QC isolates were outside the recommended range (14,16). The total of
224 SYO MICs for the 12 *Candida* species and the four triazoles pooled for ECV definition from 3 to
225 30 independent laboratories ranged from 11,171 to 17 isolates, including the data points for *C.*
226 *parapsilosis SS* and *C. parapsilosis* SC, *C. metapsilosis*, *C. orthopsilosis*, *C. famata, C. kefyr,*
227 and *S. cerevisiae*. The SYO MIC distributions for the 59 *C. albicans* and 39 *A. fumigatus SS*
228 mutants from multiple laboratories were also listed in Table 1. In the case of SYO data for
229 *Aspergillus* spp., interlaboratory modal consensus was an overall issue given that, of the
230 submitted data for five species, ECVs were only proposed for voriconazole (4 of 5 species) and
231 itraconazole (*A. niger*) (Tables 1 and 3). Of the 903 *A. fumigatus* listed in Table 1, 71% (640 data
232 points) were identified as *sensu stricto* and 29% (263 data points) as species complex
233 (identification by morphological methods, MALDI-TOF mass spectrometry, or by molecular
234 methods [e.g., β-tubulin and calmodulin sequencing]) (30). *Candida* isolates also were identified
235 to the species level by biochemical tests, MALDI-TOF mass spectrometry and/or molecular
236 methods in the laboratories submitting the data (31,32); *C. parapsilosis* and *C. glabrata* were
237 submitted mainly as SC (Table 1).

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238 Table 1 also depicts the SYO modes for *Candida* and *Aspergillus* species. The lowest
239 SYO fluconazole modes (0.25 µg/ml) were for *C. albicans*, *C. dubliniensis* and *C. kefyr* and the
240 highest mode for *C. krusei* (64 µg/ml). Similar modal diversity was noted among posaconazole
241 MICs (modes of 0.01 µg/ml for *C. albicans* and 1 µg/ml for *C. glabrata*). However, itraconazole
242 and voriconazole modes were mostly 0.06 µg/ml to 0.12 µg/ml or 0.008 to 0.03 µg/ml,
243 respectively. The exceptions were itraconazole modes for *C. glabrata* (0.5 µg/ml), *C.*
244 *guilliermondii* and *C. krusei* (0.25 µg/ml) and voriconazole modes for *C. guilliermondii*, *C.*
245 *tropicalis* (0.06 µg/ml), *C. glabrata* and *C. krusei* (0.25 µg/ml). Most SYO modes for the *C.*
246 *parapsilosis* complex were +/-1 double dilution, but all posaconazole modes for the four species
247 in the complex were 0.03 µg/ml. SYO voriconazole modes for *Aspergillus* spp. and the
248 itraconazole mode for *A. niger* ranged from 0.12 to 0.5 µg/ml. As expected, SYO modes for the
249 *C. albicans* and *A. fumigatus* mutants were much higher than those for the non-mutant isolates
250 and we observed an overlap between both groups of MICs among the lower drug
251 concentrations (Table 1). Therefore, the SYO data for *Candida* spp. showed excellent modal
252 agreement, while most SYO data points for *Aspergillus* spp. were unsuitable for the ECV
253 definition pool as previously reported among SYO posaconazole data for *A. fumigatus* (23).
254
255 Eleven of the 39 laboratories contributed Etest MICs for the four more prevalent *Candida*
256 spp., *C. krusei*, and *Aspergillus* spp. Eight laboratories, including a published study (20),
257 contributed Etest voriconazole and itraconazole data for the 75 and 81 *A. fumigatus SS*
258 mutants, respectively (Tables 2 and 5). A total of 64% (712 of the 1,112 itraconazole MICs) of *A.*
259 *fumigatus* isolates and most *Candida* isolates were identified at the species level (31,32), but *C.*
260 *glabrata* and *C. parapsilosis* mainly as species complex. Therefore, we were unable to provide
261 the potential antifungal susceptibility differences among the species in the *C. parapsilosis SC*,
262 as we did by the SYO method (Table 1). Modal variability among the Etest MIC distributions
263 entering the ECV definition data pool also precluded our ECV definition for *C. albicans* and
264 fluconazole; *C. glabrata* and both itraconazole and posaconazole; *C. parapsilosis* and
265 itraconazole and C. *krusei* and fluconazole. However, most Etest data points for the
266 *Aspergillus*/agent combinations were suitable for the ECV definition pool; although we observed
267 modal discrepancies for itraconazole and voriconazole versus *A. terreus*. Consequently, we
268 collected more suitable Etest data for *Aspergillus* spp. while the overall SYO data for *Candida*
269 spp. was superior. The lowest Etest modes were for *C. parapsilosis* versus fluconazole and
270 posaconazole (0.5 and 0.01 µg/ml, respectively), *C. tropicalis* versus itraconazole (0.03 µg/ml),
271 and *C. albicans* versus voriconazole (0.008 µg/ml). All Etest modal values for *Aspergillus* spp.

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272 ranged between 0.12 and 0.25 µg/ml, except for the itraconazole modes for *A. fumigatus* (0.5
273 µg/ml), and for *A. niger* (1 µg/ml). Etest modes for the *A. fumigatus* mutants also were much
274 higher than those for the non-mutant isolates.
275
276 Tables 3 and 4 depict the proposed ECOFFinder SYO and Etest triazole ECVs,
277 respectively, for 97.5% of the modelled MIC population for the species and triazole
278 combinations that fulfilled the CLSI criteria for ECV calculation (27). There was no need to
279 weigh the data since none of the individual distributions contributed $\geq$50% of the total. In
280 addition to SYO ECVs for the prevalent *Candida* spp., fluconazole ECVs were proposed for *C.*
281 *orthopsilosis* (4 µg/ml) and *S. cerevisiae* (16 µg/ml) (Table 3). Although fluconazole ECVs for *C.*
282 *parapsilosis* SS and SC were the same (2 µg/ml), the other ECVs for *C. parapsilosis* SS were
283 one dilution higher. To our knowledge, ECVs for *C. parapsilosis* SS or any other member of this
284 complex and for *S. cerevisiae* are not yet available for the reference methods (26,28)
285 (http://www.eucast.org/ast_of_fungi/). Due to aberrant modes by the Etest, we only defined
286 voriconazole Etest ECVs of 0.03 to 2 µg/ml for five *Candida* spp. and ECVs of 0.12 to 64 µg/ml
287 for the other three agents and 3 to 4 species (Table 4). However, we proposed ECVs for three to
288 four relevant *Aspergillus* spp. (2,4,5). Inconsistent itraconazole and voriconazole modes for *A.*
289 *terreus* from four laboratories as well as insufficient posaconazole and voriconazole MICs for *A.*
290 *nidulans* (data submitted from only two laboratories) precluded ECV definition for these two
291 species/agents (27) (data not shown in Table 2). In Table 6, we compared our SYO and Etest
292 ECVs with the approved CLSI ECVs as listed in the new edition of the M59 document (26). In
293 general, SYO ECVs were one to two dilutions higher than those for the CLSI or Etest methods.
294 In some instances, such as for fluconazole and voriconazole versus *C. glabrata,* among others,
295 SYO and CLSI ECVs of 64 and 8 µg/ml and 2 and 0.25 µg/ml, respectively, have been defined
296 (26). All these observations underscore the need for method-dependent ECVs in order to
297 properly categorize the MIC for the infecting isolate being evaluated as either WT or non-WT. It
298 also demonstrates that while commercial systems can successfully establish ‘equivalence’
299 according to FDA criteria, the pooling of data from multiple laboratories can more easily detect
300 differences between these assays and the reference method, at least in what is measured as
301 the wild type.
302
303 As mentioned above, the main role of the ECV is to identify the strains that could harbor
304 intrinsic or acquired resistance mechanisms (non-WT or mutant isolates) (27,28). CLSI MICs for
305 *Candida* and *Aspergillus* mutants are readily available in the literature (6-10,33-36), but they are

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scarce by the commercial methods (20-24). A total of 162 SYO and Etest MICs for *C. albicans*, *C. glabrata* and *C. parapsilosis* mutants were received. The number of SYO MICs above the ECVs of the four triazoles for the 59 *ERG11 C. albicans* mutants was agent-dependent. The posaconazole ECV of 0.06 µg/ml recognized the highest percentage of mutants (55/59: 93%), followed by the itraconazole ECV of 0.12 µg/ml (53/59: 90%), the voriconazole ECV of 0.01 µg/ml (52/59: 88%) and the fluconazole SYO ECV of 1 µg/ml (48/59 (81%). These *C. albicans* mutants had the following *ERG11* substitutions: F145L, Y132H, S442F, S405F, G464S, A114S, G464S, F145T, T22OL, and P98A (alone or in combination). Although high CLSI triazole MICs have been documented for most of those substitutions (6,8,33-35), T22OL and P98A (alone or in different combinations with E266D, G448R, V437I, V488I, K143R, and Y132H/X) have not been previously reported. Considering their high MICs of >8 µg/ml (Table 1), it seems that these strains also could harbor combined resistance mechanisms (e.g., the most common efflux pump overexpression +erg11 overexpression and/or mutation). These molecular combinations are due to aneuploidy (duplication of the chromosome 5 or multiplication of its long arm). However, we did not receive efflux pump overexpression data for the 59 *C. albicans* mutants. On the other hand, in Table 5 we listed the *C. albicans* and *A. fumigatus* mutants that according to our method-dependent ECVs could be categorized as either WT (MICs =< each ECV) and/or non-WT (MICs >the ECV). Those substitutions have been reported as both "susceptible and resistant" isolates using CLSI methodologies and BPs (8,33-35). Regarding data from the single laboratories, SYO MICs of the four agents for the two *C. albicans* and 11 of the 13 *C. glabrata* strains from single laboratories with overexpression of the *CDR2* and *CDR1* gene efflux pumps, respectively, were above the four ECVs (data not shown in Table 5). However, only the fluconazole and voriconazole ECVs (2 and 0.03 µg/ml, respectively) recognized >96% of the 78 *C. parapsilosis* mutants. Therefore, the potential ability of our SYO ECVs in recognizing ≥90% of the isolates with mechanisms of resistance among the most prevalent *Candida* spp. (*C. albicans, C. glabrata*) provided a preliminary indication of their clinical value. More data points for other *Candida* spp. mutants would better assess the utility of the SYO method for yeast testing in the clinical laboratory.

In the present study, a total of 75 to 81 Etest (voriconazole and itraconazole, respectively) and 39 SYO (voriconazole) MICs for *A. fumigatus* SS *cyp51A* mutants, were evaluated. Our proposed Etest itraconazole ECV of 2 µg/ml for *A. fumigatus* had a superior performance in recognizing the *cyp51A* mutants (78/81: 96%) than the voriconazole Etest ECV of 0.5 µg/ml (50/75: 67%) and the SYO ECV of 1 µg/ml (26/39: 67%) (Table 5). Etest

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340 itraconazole MICs were above the ECV for the following mutations: 48 TR34/L98 (59%), 12 G54
341 (15%), 9 M220 (11%), 5 G448S (6%) and 7 (9%) miscellaneous mutations, including two
342 TR46/Y121F (Data not listed in Table 5). However, *cyp51A* G54 changes have been linked in
343 the literature with cross-resistance to both itraconazole and posaconazole and M220 with either
344 high or low triazole MICs (36). An overlap between posaconazole MICs for non-mutants and a
345 much larger number of mutants of *A. fumigatus* by three antifungal susceptibility methods (CLSI,
346 EUCAST and Etest) also has been reported (23). These preliminary results for *Aspergillus* spp.
347 indicated that the Etest appears to be a superior method for detecting mutations in *A. fumigatus*
348 as well as for testing other *Aspergillus* spp. Once again, these results underscore the need for
349 method-dependent ECVs. As far as the SYO data for *Aspergillus* spp., further collaborative
350 studies should evaluate the endpoint determination; both color change and growth inhibition
351 have been have reported in the literature.

352

353 In conclusion, we proposed method-dependent SYO and Etest ECVs for various
354 species/triazole combinations for which suitable data were available from multiple laboratories
355 (3 to 30). Substantial data with excellent interlaboratory modal agreement were evaluated by the
356 SYO method for *Candida* and other yeasts species, including MIC distributions for the *C.*
357 *parapsilosis* complex (*C. parapsilosis* SS, *C. metapsilosis* and *C. orthopsilosis*) and *S.*
358 *cerevisiae*. Because of that, we proposed SYO ECVs for 8 to 10 yeast species and the four
359 triazoles evaluated, as well as for *C. orthopsilosis* and *S. cerevisiae* versus fluconazole. We also
360 provided MIC ranges, and more importantly, modes for other less prevalent yeast species. On
361 the other hand, interlaboratory modal agreement was better by the Etest for *Aspergillus* than for
362 yeast species. As a result, we proposed Etest ECVs of itraconazole, posaconazole and
363 voriconazole for three to four *Aspergillus* spp. and voriconazole ECVs for the four most
364 prevalent *Candida* spp. and *C. krusei*. Finally, the SYO posaconazole ECV of 0.06 µg/ml for *C.*
365 *albicans* and the Etest itraconazole ECV of 2 µg/ml for *A. fumigatus* were the best predictors in
366 recognizing the non-WT or mutants (highest percentage of MICs for mutants that were above
367 the ECV). Although ECVs of fluconazole and voriconazole for *C. parapsilosis* recognized >96%
368 of the non-WT isolates, results were unsatisfactory with posaconazole and itraconazole ECVs.
369 Data for mutants for other *Candida* spp. would better assess the method-dependent proposed
370 ECVs. The SYO method appears to yield more suitable MIC data for testing most *Candida* spp.
371 and the Etest for *Aspergillus* spp.

372

373 **Materials and Methods**

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374

375 **Isolates**: The *Candida* and other yeast isolates evaluated were recovered mostly from
376 blood and other normally sterile sites from patients with candidemia or other deep infections
377 (>90%) as well as superficial, oral, vaginal and thrush. The *Aspergillus* isolates also were
378 recovered from deep infections, sterile and other sites (mostly [>90%] bronchoalveolar lavage
379 fluids, sputum) at the following medical centers: VCU Medical Center, Richmond, VA, USA;
380 Mycology Reference Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III,
381 Majadahonda, Madrid, Spain; Unité de Parasitologie, Mycologie, Département de Bactériologie
382 Virologie Hygiène Mycologie Parasitologie, Créteil, France; Grupo de Infección Grave, Instituto
383 de Investigación Sanitaria La Fe, Valencia, Spain; Unidad de Gestión Clínica de Enfermedades
384 Infecciosas y Microbiología, Hospital de Valme, Seville, Spain; Department of Internal Medicine,
385 National Taiwan University Hospital and College of Medicine, Taipei, Taiwan; Public Health
386 Ontario, Toronto, Ontario, Canada; Klinisk Mikrobiologi, Karolinska, Universitetlaboratoriet,
387 Karolinska, Universitetssjukhuset, Stockholm, Sweden; Université Paris-Descartes, Faculté de
388 Médecine, APHP, Hôpital Européen Georges Pompidou, Unité de Parasitologie-Mycologie,
389 Service de Microbiologie, Paris, France; Laboratorio de Micología y Diagnóstico Molecular,
390 Cátedra de Parasitología y Micología, Facultad de Bioquímica y Ciencias Biológicas,
391 Universidad Nacional del Litoral, Consejo Nacional de Investigaciones Científicas y
392 Tecnológicas (CONICET), Santa Fe, Argentina; Universidad Autónoma de Nuevo León, Mexico;
393 National Institute for Communicable Diseases (Centre for Healthcare-Associated Infections,
394 Antimicrobial Resistance and Mycoses), a Division of the National Health Laboratory Service
395 and Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa;
396 Hospital General Universitario Gregorio Marañón, Madrid, Spain; SA Pathology, National
397 Mycology Reference Centre, Adelaide, S. Australia; Division of Hygiene and Medical
398 Microbiology, Medical University of Innsbruck, Innsbruck, Austria; Departamento de
399 Microbiología, Facultad de Medicina y Enfermería, Universidad de Córdoba, Córdoba, Spain;
400 Servicio de Microbilogía, Hospital Universitario Cruces, Barakaldo, Spain; Servicio de
401 Microbiología, Hospital Universitario Central de Asturias, Asturias, Spain; Departamento de
402 Inmunología, Microbiología y Parasitología, Facultad de Medicina y Enfermería, Universidad del
403 País Vasco/Euskal Herriko Unibertsitatea, UPV/EHU, Bilbao, Spain; Departamento de
404 Biomedicina, Biotecnología y Salud Pública, Universidad de Cádiz, Cadiz, Spain; Hospital de
405 Alcañiz, Alcañiz (Teruel), Spain; Hospital de la Santa Creu i Sant Pau, Barcelona, Spain;
406 Institute of Microbiology, Università Cattolica del Sacro Cuore, Rome, Italy; University of
407 Pittsburgh, Pittsburgh, Pennsylvania, USA; Department of Pharmaceutical Technology and

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408 Biochemistry, Faculty of Chemistry, Gdańsk University of Technology, Gdańsk, Poland;
409 Department of Pharmaceutical Technology and Biochemistry, Faculty of Chemistry, University of
410 Technology, Gdansk, Poland; Department of Biomedical Sciences for Health, Università degli
411 Studi di Milano, Milan, Italy; Mayo Clinic, Rochester, MN, USA; Microbiology Laboratory,
412 Ospedale San Gerardo, Monza, Italy; Microbiology and Virology Unit IRCCS Policlinico San
413 Matteo, Pavia, Italy; Microbiology Section, Humanitas Research Hospital, Milan, Italy;
414 Microbiology Laboratory, A.O. Spedali Civili, Brescia, Italy; Microbiology Laboratory, Fondazione
415 IRCCS Cà Granda O. Maggiore Policlinico, Milan, Italy; Microbiology Laboratory, Niguarda
416 Hospital, Milan, Italy; Clinical Microbiology Laboratory, Attikon Hospital, Medical School,
417 National and Kapodistrian, University of Athens, Athens, Greece; Laboratório Especial de
418 Micologia, Disciplina de Infectologia, Escola Paulista de Medicina, Universidade Federal de São
419 Paulo, São Paulo, SP, Brazil; Microbiology Institute, ASST 'Papa Giovanni XXIII, Bergamo, Italy;
420 Unidad de Micología, Servicio de Microbiología, Hospital Universitario La Fe, Valencia, Spain;
421 Microbiology-ASST Lariana, Como, Italy; and Medicina di Laboratorio, IRCCS Policlinico San
422 Donato, Milan, Italy.

423

424 The total of submitted triazole MICs of the four triazoles by both SYO and/or Etest
425 methods from 3 to 30 laboratories for yeast species were as follows (method-agent dependent)
426 (Tables 1 and 2): 11,171 *C. albicans*, 215 *C. dubliniensis*, 4,418 *C. glabrata* SC (including 349
427 *C. glabrata* SS), 157 *C. guilliermondii*, 676 *C. krusei*, 298 *C. lusitaniae*, 3,691 *C. parapsilosis*
428 SC, 922 *C. parapsilosis* SS, and 1,854 *C. tropicalis* isolates were evaluated for ECV definition
429 (Tables 1 and 2). SYO MICs for other less common *Candida* and yeast species from at least
430 three laboratories were collected for 25 *C. famata*, 55 *C. kefyr,* 36 *C. metapsilosis*, and 110 *C.*
431 *orthopsilosis* as well as SYO data for 244 isolates of *S. cerevisiae*. In addition, we pooled SYO
432 and mostly Etest data for the four most prevalent *Aspergillus* complexes as follows (method-
433 agent dependent): 1,409 *A. fumigatus*, 389 *A. flavus*, 103 *A. nidulans*, 233 *A. niger*, and 302 *A.*
434 *terreus* isolates originating from 3 to 11 independent laboratories.

435

436 We also received a total of 282 MICs for mutants: 59 SYO and 10 Etest MICs,
437 respectively, for *C. albicans* (*Erg11* gene mutations), 2 *C. albicans* and 13 *C. glabrata*,
438 respectively, (overexpression of CDR2 or CDR1 efflux pumps, respectively), and 78 *C.*
439 *parapsilosis* (*Erg11* and *MRR1*). SYO and Etest MICs were gathered for 39 and 81 strains for *A.*
440 *fumigatus* SS mutant isolates, respectively, with *cyp51A* gene mechanisms of resistance
441 (TR34/L98H, G54, M220, and others) from five to seven participant laboratories and one

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442 previous Etest study (20) (Tables 1-2 and Table 5). The isolates were identified at each medical
443 center by conventional and molecular methodologies that included macro-and microscopic
444 morphology, thermotolerance (incubation at 50°C), MALDI-TOF and β-tubulin and calmodulin
445 sequencing (31,32). Since molecular identification was not performed for all the isolates
446 evaluated in the present study, we listed the non-mutant isolates in the respective Tables as the
447 complexes of *C. glabrata* or *C. parapsilosis* or *Aspergillus* spp. Strains of *A. fumigatus*, *C.*
448 *albicans* and *C. glabrata* that were submitted as having mutations were screened in the
449 participant laboratories using published protocols. (31,36-38).
450
451 At least one of following quality control (QC) isolates: *C. parapsilosis* ATCC 22019, *C.*
452 *krusei* ATCC 6258 and *Paecilomyces variottii* ATCC MYA-3630 and/or reference isolates *A.*
453 *fumigatus* ATCC MYA-3626 and *A. flavus* ATCC MYA-204304 were evaluated by the two
454 methods in each of the participant laboratories (14,16). MIC data were not included in the study
455 unless the participant laboratories reported that their MICs for the individual QC isolates used in
456 each center were within the expected MIC ranges.
457
458 **Antifungal susceptibility testing**. Triazole SYO and Etest MICs were obtained by the
459 two commercial antifungal susceptibility methods by following the manufacturer's guidelines (14-
460 16). The SYO MIC was the first blue or purple well after 24 h (*Candida*) or mostly 48 h
461 (*Aspergillus*) of incubation and isolates, respectively). The Etest MIC was the lowest drug
462 concentration at which the border of the growth-free elliptical inhibition intercepted the scale on
463 the antifungal strip, after 24 to 48 h, as needed; trailing growth was allowed solely for the
464 definition of Etest MICs for *Candida* isolates.
465
466 **Definitions.** The definition of the ECV as a categorical endpoint has been widely
467 described as well as above (27,28). Briefly, the ECV is the highest MIC/MEC distribution of the
468 WT population and is established by using reliable MIC/MEC distributions from at least three
469 laboratories. A non-WT organism usually shows reduced susceptibility to the agent being
470 evaluated compared to the WT (no phenotypic resistance) population. In addition to MIC
471 distributions, the ECV calculation takes into account each laboratory distribution mode, the
472 inherent variability of the test (usually within one doubling dilution), and that the ECV should
473 encompass 95 to 97% of isolates. We used those same criteria and requirements for
474 establishing our proposed Etest and SYO method-dependent ECVs. Most published ECVs are

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475 based on reference MIC distributions, and ECVs based on other methods could be different, as
476 it has been shown in our study (Table 6).
477
478 **Data collation and analyses.** Triazole MICs were submitted from 39 independent
479 worldwide laboratories (method/agent/species dependent) in order: (i) to define MIC
480 distributions by each commercial susceptibility testing method/agent and species; (ii) to examine
481 the suitability of these distributions for pooling prior to ECV setting, including the evaluation of
482 interlaboratory modal agreement; and (iii) to estimate ECVs for each species/agent/method that
483 fulfilled the CLSI criteria for ECV definition after pooling (at least 100 MICs for each
484 species/method/agent that originated in $\geq$3 independent laboratories) (27,28). ECVs were
485 estimated by the iterative statistical method at the 97.5% cutoff value (29) or the second
486 numerical derivative method when the putative wild-type mode was at the lowest concentration
487 in the distribution (30) (Tables 2 and 4). SYO MIC distributions for less common yeast species
488 (*C. famata* and *C. kefyr*) and *C. metapsilosis* also were reported when they originated from at
489 least three laboratories and had comparable modes.
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495
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Table 1. SYO pooled triazole MIC distributions for species of *Candida*, *Saccharomyces* and *Aspergillus*[a]

Agent and species	No. isolates	No. labs used/ Total[b]	Number of isolates with MIC (µg/ml) of:[c]											
Fluconazole			**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**≥128**
C. albicans	11,171	28/30	12	1,016	**4,252**	4,152	978	238	122	82	78	33	49	159
Confirmed *ERG11 mutants*	59	4/4		3	1	4	3		1	2	2		**43**	
C. dubliniensis	195	7/10		48	**64**	57	13	6	3	2		1	1	
C. famata	23	3/6			1	2	**11**	5	3	1				
C. glabrata	4,418	30/30		13	9	23	64	152	375	1,049	**1,330**	691	216	496
C. guilliermondii	153	8/13		2	1	6	20	36	**46**	19	10	6	4	3
C. kefyr	55	3/4		13	**25**	15	2							
C. krusei	537	15/16		1	1	1			3	8	43	193	**220**	67
C. lusitaniae	298	12/12		16	41	75	**99**	43	12	5	1	4	1	1
C. parapsilosis	3,691	28/30		89	502	**1,210**	958	421	221	151	82	27	19	11
C. parapsilosis SS	911	5/5		18	118	**282**	216	121	74	53	19	6	4	
C. metapsilosis	36	4/4			1	2	**17**	10	5		1			
C. orthopsilosis	110	5/5		3	4	29	**43**	15	8	4	2		1	1
C. tropicalis	1,854	24/28		20	82	270	**701**	482	129	53	19	24	14	60
S. cerevisiae	244	3/3		4	3	9	40	70	**76**	26	10	4	2	
Itraconazole			**0.008**	**0.01**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**≥16**
C. albicans	7,843	27/30	69	995	2,696	**2,754**	905	164	77	27	14	7	9	126
Confirmed *ERG11 mutants*	59	4/4			2	2	2	5	11	3	7	4		**23**

C. dubliniensis	125	6/8		13	21	**47**	27	7	2	5	1			2
Itraconazole (Cont.)			**0.008**	**0.01**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**≥16**
C. famata	18	3/5		1	1	2	3	**7**	3	1				
C. glabrata	3,594	29/30		12	19	42	112	428	**1,335**	910	195	71	26	444
C. guilliermondii	149	9/13		3		8	31	**55**	37	10	2			3
C. kefyr	45	3/3		5	10	**17**	12	1						
C. krusei	574	13/16		4	3	14	69	**283**	156	33	2	1		9
C. lusitaniae	171	8/11		11	12	52	**60**	28	7	1				
C. parapsilosis	3,353	23/30		209	570	1,098	**1,150**	252	59	13	1	1		
C. parapsilosis SS	730	4/5		68	79	237	**254**	83	6	3				
C. metapsilosis	32	4/4		3	3	**12**	11	1	2					
C. orthopsilosis	88	3/4		2	13	**35**	26	12						
C. tropicalis	1,399	23/29		14	51	138	**513**	508	126	16	4	1	5	23
S. cerevisiae	41	3/3		1	1	3	**21**	11	2	2				
A. niger	233	6/7		18	23	48	**69**	44	17	6			1	7
Posaconazole			**0.008**	**0.01**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**≥16**
C. albicans	6,729	27/30	596	**2,768**	2,318	587	175	96	56	32	10	3	60	28
Confirmed *ERG11* *mutants*	59		1		3	1	4	9	8	7	1	1		**24**
C. dubliniensis	185	7/8	35	56	**63**	25	4			2				
C. glabrata	2,999	25/29	4	5	28	39	50	153	590	**1,145**	579	62	251	93
C. guilliermondii	111	9/12	3	1	9	15	27	**35**	18	3				
C. kefyr	40	3/3			7	**13**	13	5	2					
C. krusei	562	13/15	1	1	3	20	90	**264**	151	25	5		2	

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C. lusitaniae	172	11/11	17	49	**58**	36	10		1	1				
Posaconazole (Cont.)			**0.008**	**0.01**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**≥16**
C. parapsilosis	3,085	26/30	136	538	**1,091**	915	297	86	11	7	1	1		2
C. parapsilosis SS	670	5/5	40	127	**206**	193	69	31	2				2	
C. metapsilosis	17	3/4		3	**7**	4	3							
C. orthopsilosis	30	3/4		5	**14**	7	4							
C. tropicalis	1,366	23/29	16	50	107	250	**408**	336	147	22	6		17	7
S. cerevisiae	41	3/3				3	6	**20**	9	2	1			
Voriconazole			**0.008**	**0.01**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**≥16**
C. albicans	8,747	29/30	**5,947**	1,691	481	222	111	82	47	22	10	15	76	43
Confirmed *ERG11* mutants	59	4/4	3	4	4	1	4	1	7	6	3	5	2	**19**
C. dubliniensis	215	7/9	**182**	21	5	3	1	2			1			
C. famata	25	3/5	5	**10**	4	2	2	2						
C. glabrata	3,255	24/30	23	29	65	189	486	**911**	824	340	136	156	82	14
C. guilliermondii	157	11/12	8	10	32	**46**	34	10	11	4	1			1
C. kefyr	55	3/3	**46**	8	1									
C. krusei	676	14/16	2	1	1	16	108	**291**	199	42	11	3	1	1
C. lusitaniae	248	11/12	**120**	70	32	15	4	1	2	4				
C. parapsilosis	2,670	26/30	**1,213**	695	364	210	103	50	16	10	9			
C. parapsilosis SS	718	5/5	**261**	185	122	80	47	12	5	4	2			
C. metapsilosis	30	3/4	2	10	**11**	4	2	1						
C. orthopsilosis	20	3/4	1	**8**	3	6	2							
C. tropicalis	1,637	19/28	45	92	227	**466**	443	200	70	25	20	9	23	17
S. cerevisiae	41	3/3	1	3	**17**	15	2	2	1					

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A. fumigatus	903	8/8	2	7	35	64	157	**396**	179	33	8	7	7	8
Confirmed *Cyp51A mutants*	39	5/5					3	4	8	3	4	5	2	10
Voriconazole (Cont.)			**0.008**	**0.01**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**≥16**
A. flavus	389	6/7	5	1	14	32	89	**139**	59	29	16	1	0	4
A. niger	74	3/6				1	9	19	**33**	12				
A. terreus	302	5/6	6	5	16	19	48	**122**	69	15	2			

[a]Including the complexes of *C. glabrata, C. parapsilosis* and *Aspergillus* spp.; the *cyp51A* mutants are *A. fumigatus* SS; *C. famata* (*D. hansenii*), *C. guilliermondii* (*M. guilliermondii*), *C. kefyr* (*K. marxianus*), *C. krusei* (*P. kudriavzevii*) and *C. lusitaniae* (*Clavispora lusitaniae*).

[b]Total number of laboratories included in the ECV definition pool/total number of laboratories that submitted data.

[c]Data are from between 3 and 30 laboratories determined by the colorimetric broth microdilution SYO method (14); the highest number in each row (showing the most frequent MIC or the mode) is in bold.

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Table 2. Etest Triazole pooled MIC distributions for species of *Candida* and *Aspergillus*[a]

Agent and species	No. isolates	No. labs used/ total[b]	Number of isolates with MIC (µg/ml) of:[c]												
Fluconazole			**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**≥128**
C. glabrata	356	7/10			1	4	13	34	50	79	**88**	36	13	28	10
C. parapsilosis	639	9/9	3	19	68	131	**153**	138	66	21	9	7	20		4
C. tropicalis	368	9/10	3	5	11	61	96	**120**	47	11	4	1	4	4	1
Itraconazole			**≤0.004**	**0.008**	**0.01**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**≥16**
C. albicans	975	8/9	7	55	145	237	**295**	150	27	19	16	6	7	5	6
C. krusei	101	3/3				1		2	9	**36**	35	7	7	1	3
C. tropicalis	165	5/8	2	12	23	**39**	30	22	15	11	7	2	1	1	
A. fumigatus	1,112	10/10		4	1	3	30	56	157	**483**	268	73	12	5	20
Confirmed *Cyp 51A* mutants	81	8/8					1			1		1	7	4	**67**
A. flavus	250	7/8			1	4	19	37	**103**	69	16	1			
A. nidulans	130	4/4			1	1	13	**39**	34	23	11	7		1	
A. niger	176	4/5				1	1	2	5	25	**71**	45	17	5	4
Posaconazole			**≤0.004**	**0.008**	**0.01**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**≥16**
C. albicans	305	4/6	6	29	94	**102**	44	17	9	3	1				
C. krusei	48	3/3					1	5	**17**	16	7				2
C. parapsilosis	162	4/5	8	26	**51**	37	23	9	3	2			1		2
C. tropicalis	101	4/5		9	21	**32**	21	4	8	3	1		1	1	
A. flavus	204	7/7			1	4	14	70	**96**	17	2				
A. niger	168	4/5				5	16	58	**73**	15	1				
A. terreus	194	5/5				8	47	**105**	27	4	2		1		

Voriconazole (Cont. Table 2)			**≤0.004**	**0.008**	**0.01**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**≥16**
C. albicans	2,159	8/11	485	**803**	491	158	104	42	22	20	11	5	7	1	**10**
C. glabrata	551	8/9		7	11	20	37	105	**143**	96	63	30	15	14	10
C. krusei	130	6/6			1	2	3	20	30	**45**	24	5		2	
C. parapsilosis	506	7/9	4	20	46	97	**167**	100	39	15	4	4	8	1	1
C. tropicalis	260	6/10	4	4	12	28	82	**88**	22	14	5			1	
A. fumigatus	1,409	11/11	1	6	2	30	132	**633**	473	100	19	7	3	2	1
Confirmed *Cyp 51A* mutants	75	8/8					5	6	7	8	13	15	3	1	**17**
A. flavus	257	7/7			1		18	84	**103**	39	10	1			1
A. niger	173	4/5			2	3	22	37	**81**	25	1	1			1

[a]Including the complexes of *C. parapsilosis*, *C. glabrata* and *Aspergillus* species; the *Cyp 51A* mutants are *A. fumigatus* SS; *C. krusei* (*P. kudriazveii*).

[b]Total number of laboratories included in the ECV definition pool/total number of study laboratories and one published study (20) that submitted data.

[c]Data are from between 3 and 11 laboratories and were determined by the agar diffusion Etest method (15); the highest number in each row (showing the most frequent MIC or the mode) is in bold.

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Table 3. Method-dependent SYO ECOFFinder ECVs of four triazoles for species of *Candida, Saccharomyces*, and *Aspergillus*[a]

Agent and species	No. isolates	No. labs used/total	MIC (µg/ml) Range	MIC (µg/ml) Mode	ECVs (µg/ml)[b]
Fluconazole					
C. albicans	11,171	28/30	0.06-≥128	0.25	1
C. dubliniensis	195	7/10	0.12-64	0.25	1
C. glabrata	4,418	30/30	0.12-≥128	16	64
C. guilliermondii	153	8/13	0.12-≥128	4	16
C. krusei	537	15/16	0.12-≥128	64	128
C. lusitaniae	298	12/12	0.12-≥128	1	4
C. parapsilosis	3,691	28/30	0.12-≥128	0.5	2
C. parapsilosis SS	911	5/5	0.12-64	0.5	2
C. orthopsilosis	110	5/5	0.12-≥128	1	4
C. tropicalis	1,854	24/28	0.12-≥128	1	4
S. cerevisiae	244	3/3	0.12-64	4	16
Itraconazole					
C. albicans	7,843	27/30	0.008-≥16	0.06	0.12
C. dubliniensis	125	6/8	0.01-≥16	0.06	0.25
C. glabrata	3,594	29/30	0.01-≥16	0.5	2
C. guilliermondii	149	9/13	0.01-≥16	0.25	1
C. krusei	574	13/16	0.01-≥16	0.25	1
C. lusitaniae	171	8/11	0.01-1	0.12	0.5
C. parapsilosis	3,353	23/30	0.01-4	0.12	0.25
C. parapsilosis SS	730	4/5	0.01-1	0.12	0.5
C. tropicalis	1,399	23/29	0.01-≥16	0.12	0.5
A. niger	233	6/7	0.01-≥16	0.12	1
Posaconazole					
C. albicans	6,729	27/30	0.008-≥16	0.01	0.06
C. dubliniensis	185	7/8	0.008-1	0.03	0.12
C. glabrata	2,999	25/29	0.008-≥16	1	4
C. guilliermondii	111	9/12	0.008-1	0.25	1
C. krusei	562	13/15	0.008-8	0.25	1
C. lusitaniae	172	11/11	0.008-1	0.03	0.12
C. parapsilosis	3,085	26/30	0.008-≥16	0.03	0.12
C. parapsilosis SS	670	5/5	0.008-8	0.03	0.25
C. tropicalis	1,366	23/29	0.008-≥16	0.12	1
Voriconazole					

(Cont. Table 3)

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C. albicans	8,747	29/30	0.008-≥16	0.008	0.01[c]
C. dubliniensis	215	7/9	0.008-2	0.008	0.01[c]
C. glabrata	3,255	24/30	0.008-≥16	0.25	2
C. guilliermondii	157	11/12	0.008-≥16	0.06	0.5
C. krusei	676	14/16	0.008-≥16	0.25	1
C. lusitaniae	248	11/12	0.008-1	0.008	0.03[c]
C. parapsilosis	2670	26/30	0.008-2	0.008	0.01[c]
C. parapsilosis SS	718	4/5	0.008-2	0.008	0.03[c]
C. tropicalis	1,637	19/28	0.008-≥16	0.06	0.5
A. fumigatus	903	8/8	0.008-≥16	0.25	1
A. flavus	389	6/7	0.008-≥16	0.25	1
A. terreus	302	5/6	0.008-2	0.25	1

[a]Including the complexes of *C. parapsilosis*, *C. glabrata* and *Aspergillus* species; *C. guilliermondii* (*M. guilliermondii*), *C. krusei* (*P. kudriavzevii*) and *C. lusitaniae* (*Clavispora lusitaniae*. Modal variability or insufficient data precluded the proposal of ECVs for some species of both *Candida* and *Aspergillus*.

[b]ECOFFinder ECVs for 97.5% of the statistically modelled population based on MICs by the colorimetric broth microdilution SYO method (14,29) except where indicated by superscript c, referring to footnote c. Proposed method-dependent SYO ECV for *A. fumigatus* and posaconazole is 0.06 µg/ml, as reported elsewhere (23). *C. krusei* is intrinsically resistant to fluconazole regardless of the MIC.

[c]ECV as estimated using the second derivative method (30).

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Table 4. Method-dependent Etest ECOFFinder ECVs of four triazoles for species of *Candida* and *Aspergillus*[a]

Agent and species	No. isolates	No. labs used/total[b]	MIC (µg/ml)		ECVs (µg/ml)[c]
			Range	Mode	
Fluconazole					
C. glabrata	356	7/10	0.12-≥128	8	64
C. parapsilosis	639	9/9	0.03-≥128	0.5	4
C. tropicalis	368	9/10	0.03-≥128	1	4
Itraconazole					
C. albicans	975	8/9	≤0.004-≥16	0.06	0.25
C. krusei	101	3/3	0.03-≥16	0.5	2
C. tropicalis	165	5/8	≤0.004-8	0.03	0.5
A. fumigatus	1,112	10/10	0.008-≥16	0.5	2
A. flavus SC	250	7/8	0.01-2	0.25	1
A. nidulans	130	4/4	0.01-8	0.12	1
A. niger	176	4/5	0.03-≥16	1	4
Posaconazole[c]					
C. albicans	305	4/6	≤0.004-1	0.03	0.12
C. parapsilosis	162	4/5	≤0.004-≥16	0.01	0.12
C. tropicalis	101	4/5	0.008-8	0.03	0.12
A. flavus	204	7/7	0.01-1	0.25	0.5
A. niger	168	4/5	0.03-1	0.25	0.5
A. terreus	194	5/5	0.03-4	0.12	0.25
Voriconazole					
C. albicans	2,159	8/11	≤0.004-≥16	0.008	0.03
C. glabrata	551	8/9	0.008-≥16	0.25	2
C. krusei	130	6/6	0.01-8	0.5	2
C. parapsilosis	506	7/9	≤0.004-≥16	0.06	0.25
C. tropicalis	260	6/10	≤0.004-8	0.12	0.5
A. fumigatus	1,409	7/7	≤0.004-≥16	0.12	0.5
A. flavus	257	7/7	0.01-≥16	0.25	0.5
A. niger	173	4/5	0.01-≥16	0.25	1

[a]Including the complexes of *C. parapsilosis*, *C. glabrata* and *Aspergillus* spp.; *C. krusei* (*P. kudriazveii*). Variability or insufficient data precluded the proposal of ECVs for some species of both *Candida* and *Aspergillus*.

[b]Total number of laboratories included in the ECV definition pool/total number of laboratories that submitted data (including data from one published study) (20).

[c]ECVs for 97.5% of the statistically modelled population by ECOFFinder calculations and based on MICs by the commercial agar diffusion Etest method (15,29). Proposed method-dependent Etest ECV for *A. fumigatus* and posaconazole was 0.25 µg/ml, as reported elsewhere (23).

Table 5. Triazole SYO and Etest MICs for selected confirmed *C. albicans ERG11* and *A. fumigatus sensu stricto cyp51* mutants[a]

Species/agent	Mutation/ Method	No. of mutants with MIC (µg/ml) of:[b]							Total mutants ≤ECV[b]
Fluconazole	**SYO**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**≥8**	
C. albicans	E266D			1	2			7	3
	E266D/V4881				1			7	1
	V112I/G450R			1				3	1
	K128T			2				1	2
	D116E/K128T/ V159I	3	1						4
									11/59
Itraconazole	**SYO**	**≤0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**≥8**	
C. albicans	E266D		1					7	2
	E266D/V4881		1					7	1
	V112I/G450R	1			4			1	1
	D116E/K128T/ V159I	3						1	3
									6/59
Posaconazole	**SYO**	**≤0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**≥8**	
C. albicans	V112I/G450R	1			2	1			1
	D116E/K128T/ V159I	3						1	3
									4/59
Voriconazole	**SYO**	**≤0.01**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**≥1**	
C. albicans	E266D	1	1		2			6	1
	E266D/V4881	1						7	1
	K128T	1	1					1	1
	D116E/K128T	4						4	4
									7/59
Itraconazole	**Etest**	**≤0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**≥8**	
A. fumigatus	G448S				1			4	1
	M220K						1		1
	I301T	1						1	1
									3/81
Voriconazole	**Etest**	**<0.06**	**0.12**	**0.2**	**0.5**	**1**	**2**	**≥4**	
A. fumigatus	TR34		1		2	14	11	10	3
	G54E/R/W	2	2	5	3				12
	M220I/K//R/T/ V		3	1	4		2	1	8
	G138C			1					1
	I301T	1							1
									25/75

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(Cont. Table 5)

Voriconazole	**SYO**	**<0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**≥4**	
A. fumigatus	TR34					1			1
	G54E/R/W		2	1	2				5
	M220I/K/T/V			2	3				5
	G138C			1					1
	I301T		1						1
									13/39

[a]Listed are SYO and Etest MICs for *C. albicans* and *A. fumigatus* mutants that were either below and/or above (shaded and non-shaded, respectively) each correspondent ECV among the total data points for the 59 *C. albicans* and 75 or 81 or 39 *A. fumigatus* mutants. Data submitted from multiple participant laboratorios (4 to 8) and a single published study (20).

[b]The proposed SYO ECVs were: *C. albicans* versus fluconazole (1 µg/ml), itraconazole (0.12 µg/ml), posaconazole (0.06 µg/ml) and voriconazole (0.01 µg/ml); and for *A. fumigatus* and voriconazole (1 µg/ml). Etest ECVs were: *A. fumigatus* versus itraconazole (2 µg/ml) and voriconazole (0.5 µg/ml).

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Table 6. Method-dependent ECVs of four triazoles for species of *Candida*, *Saccharomyces*, and *Aspergillus* by three susceptibility testing methods[a]

Species	Agent/ Method-dependent ECVs (µg/ml)											
	FLU			ITR			POS			VOR		
	SYO	Etest	CLSI	SYO	Etest	CLSI	SYO	Etest	CLSI	SYO	Etest	CLSI
C. albicans	1	AM	0.5	0.12	0.25	NA	0.06	0.12	0.06	0.01	0.03	0.03
C. dubliniensis	1	ID	0.5	0.25	ID	NA	0.12	ID	0.25	0.01	ID	0.03
C. glabrata	64	64	8	2	8	4	4	ID	1	2	2	0.25
C. guilliermondii	16	ID	8	1	ID	NA	1	ID	0.5	0.5	ID	0.12
C. krusei	128	ID	32	1	2	1	1	ID	0.5	1	2	0.5
C. lusitaniae	4	ID	1	0.5	ID	0.5	0.12	ID	0.06	0.03	ID	0.06
C. parapsilosis SC	2	4	1	0.25	AM	NA	0.12	0.12	0.25	0.01	0.25	0.03
C. parapsilosis SS	2	NA	NA	0.5	NA	NA	0.25	NA	NA	0.03	NA	NA
C. tropicalis	4	4	1	0.5	0.5	0.5	1	0.12	0.12	0.5	0.5	0.12
S. cerevisiae	16	ID	NA	ID	ID	NA	ID	ID	NA	ID	ID	NA
A. fumigatus	NA	NA	NA	AM	2	1	0.06[b]	0.25[b]	0.25[b]	1	0.5	1
A. flavus	NA	NA	NA	AM	1	1	NA	0.5	0.5	1	0.5	2
A. niger	NA	NA	NA	1	4	4	NA	0.5	2	ID	1	2
A. terreus	NA	NA	NA	AM	AM	2	NA	0.25	1	1	AM	2

[a]SYO/Etest proposed ECVs in the present study, based on MICs determined by both commercial, respectively, and CLSI broth microdilution (M27 and M38) methods (11,12,14,15). *C. guilliermondii* (*M. guilliermondii*), *C. krusei* (*P. kudriavzevii*) and *C. lusitaniae* (*Clavispora lusitaniae*).

[b]Posaconazole ECVs for *A. fumigatus* as reported elsewhere (23,26); the SYO ECV for *C. orthopsilosis* was 4 µg/ml.

AM: aberrant modes, modal variability; ID, insufficient number of laboratories/isolates entering the ECV definition pool.

NA: not available or applicable.

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