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MAJOR ARTICLE

Global Emergence of Resistance to Fluconazole and Voriconazole in *Candida parapsilosis* in Tertiary Hospitals in Spain During the COVID-19 Pandemic

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Background. *Candida parapsilosis* is a frequent cause of candidemia worldwide. Its incidence is associated with the use of medical implants, such as central venous catheters or parenteral nutrition. This species has reduced susceptibility to echinocandins, and it is susceptible to polyenes and azoles. Multiple outbreaks caused by fluconazole-nonsusceptible strains have been reported recently. A similar trend has been observed among the *C. parapsilosis* isolates received in the last 2 years at the Spanish Mycology Reference Laboratory.

Methods. Yeast were identified by molecular biology, and antifungal susceptibility testing was performed using the European Committee on Antimicrobial Susceptibility Testing protocol. The *ERG11* gene was sequenced to identify resistance mechanisms, and strain typing was carried out by microsatellite analysis.

Results. We examined the susceptibility profile of 1315 *C. parapsilosis* isolates available at our reference laboratory between 2000 and 2021, noticing an increase in the number of isolates with acquired resistance to fluconazole, and voriconazole has increased in at least 8 different Spanish hospitals in 2020–2021. From 121 recorded clones, 3 were identified as the most prevalent in Spain (clone 10 in Catalonia and clone 96 in Castilla-Leon and Madrid, whereas clone 67 was found in 2 geographically unrelated regions, Cantabria and the Balearic Islands).

Conclusions. Our data suggest that concurrently with the coronavirus disease 2019 pandemic, a selection of fluconazole-resistant *C. parapsilosis* isolates has occurred in Spain, and the expansion of specific clones has been noted across centers. Further research is needed to determine the factors that underlie the successful expansion of these clones and their potential genetic relatedness.

Keywords. *Candida parapsilosis*; antifungal resistance; fluconazole; outbreaks; voriconazole.

Candida parapsilosis is an opportunistic pathogenic yeast able to cause invasive diseases such as candidemia. Worldwide, it is the third cause of blood yeast infection after *C. albicans* and *C. glabrata*, although in some countries its incidence is higher than *C. glabrata* [1–4]. Neonates, as well as indwelling parenteral nutrition and central nervous catheters, have been associated

Received 03 June 2022; editorial decision 01 November 2022; accepted 03 November 2022; published online 7 November 2022

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https://doi.org/10.1093/ofid/ofac605

with a higher risk of infection [5, 6]. Besides sporadic infections, *C. parapsilosis* is well known to cause nosocomial outbreaks through direct and indirect contact via the hands of health care workers and through contaminated patient care equipment.

Candida parapsilosis exhibits a reduced natural in vitro susceptibility to echinocandins [7], so the main therapeutic options for invasive infections due to this species are the triazoles, mainly fluconazole or, alternatively, polyenes. Acquired resistance to fluconazole in *C. parapsilosis* is a rare phenomenon, <5% of isolates in different epidemiological studies [2, 7–10]. In recent years, however, a steady increase in resistance has been observed worldwide, mostly in the context of nosocomial outbreaks [11–20]. In many cases, these outbreaks are monoclonal and are associated with mutations in *ERG11* (mainly with the Y132F mutation), overexpression of efflux pumps (as Mdr1 and Cdr1), and mutations in *MRR1*, which encodes a transcription factor that regulates the expression of some efflux pumps [11–13, 16, 18, 21, 22].

The National Centre for Microbiology from Instituto de Salud Carlos III (CNM-ISCIII, Madrid, Spain) acts as a national reference center for clinically isolated fungi, providing services such as genotyping and confirmation of antifungal susceptibility profiles by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standardized methodology. Since 2020, a significant increase in the number of fluconazole-nonsusceptible (FNS) *C. parapsilosis* isolates received was noted, most of them coming from tertiary hospitals across the country reporting strong epidemiological suspicion of ongoing outbreaks.

The aim of this work was to describe the antifungal susceptibility profile of all the *C. parapsilosis* isolates received in the Spanish Mycology Reference Laboratory (SMRL) to gain insights about the susceptibility profile and appearance of resistance in this species. Typing analysis confirmed genetic relatedness between isolates and their distribution across centers not always in the geographical vicinity.

This nationwide study adds new data to the worrisome phenomenon of worldwide emerging azole resistance in *C. parapsilosis* and points toward a temporal relationship between the coronavirus disease 2019 (COVID-19) pandemic and the expansion of clonal outbreaks in several Spanish tertiary hospitals.

METHODS

Media and Strains Identification

The isolates were primarily isolated, identified, and screened for fluconazole non-susceptibility at local laboratories following the routine methodologies of each center. Isolates sent to the CNM-ISCIII from 2000 to 2021 and identified as *C. parapsilosis* sensu stricto were subcultivated onto Sabouraud solid or liquid medium. Identification was confirmed by sequencing the internal transcribed spacer 1 (ITS1) and ITS2 regions from the ribosomal DNA as previously described [23].

Table 1. Oligonucleotides Designed to Sequence the *Candida parapsilosis ERG11* Gene

Oligonucleotide Name	Sequence (5′- 3′)
01_F_CpERG11	CGTCAAATGTCAGCATCGTC
02_R_CpERG11	TCATTCGAGGTGAGTCAAC
03_F_CpERG11	TGGGTTGGTTCAGCCGTATC
05_F_CpERG11	ACCATCTTCACTGCATCTAG
07_F_CpERG11	GTTGCATTTGGCTGAGAAGC
09_F_CpERG11	CCAAAGGTGTTAGCTCTTCG
10_R_CpERG11	GACATAGGCAAACTGTTCACC
08_R_CpERG11	CCACCTTTACCAGATAAGGC
06_R_CpERG11	GCATACAATTGAGCAAATGAAGC
04_R_CpERG11	CCAAGTACACCGTCATTACTC

Abbreviations: F, forward; R, reverse.

Antifungal Susceptibility

Antifungal susceptibility testing was performed following the EUCAST protocol [24] (RPMI 1640 medium; Merck, Sigma-Aldrich). The medium was buffered with MOPS (Merck, Sigma-Aldrich) at pH 7 and supplemented with 2% glucose (Merck, Sigma-Aldrich). The following antifungals were tested in the concentration range indicated in brackets: amphotericin B (AmB; Merck, Sigma-Aldrich, 16–0.03 mg/L), flucytosine (64–0.125 mg/L), fluconazole (FLC; Merck, Sigma-Aldrich, 64–0.125 mg/L), itraconazole (ITZ; Janssen Pharmaceutical Research and Development, 8–0.016 mg/L), voriconazole (VOR; Pfizer Pharmaceutical Group, 8–0.016 mg/L), posaconazole (POS; Merck, Sigma-Aldrich, 8–0.016 mg/L), isavuconazole (ISV; Pfizer Pharmaceutical Group, 8–0.016 mg/L), caspofungin (CSP; Merck, Sigma-Aldrich, 16–0.016 mg/L), micafungin (MICA; Astellas Pharma Inc, 2–0.004 mg/L), and anidulafungin (ANID; Pfizer Pharmaceutical Group, 4–0.008 mg/L). The minimal inhibitory concentration (MIC) was defined as the concentration that caused 50% of growth inhibition compared with the control well without an antifungal, except for amphotericin B (90%). Strains were categorized as susceptible (S), resistant (R), or intermediate or susceptible increased exposure (I) following the breakpoints established by EUCAST (see https://www.eucast.org/astoffungi/clinicalbreakpointsforantifungals, document from February 4, 2020) [24]. Control strains *C. parapsilosis* ATCC 22 019 and *C. krusei* ATCC 6258 were included in all the assays.

Sequencing of the *ERG11* Gene

To identify mutations at the *ERG11* gene, we designed different primers (Table 1). The whole gene was amplified using oligonucleotides 01_F and 02_R using the following polymerase chain reaction (PCR) conditions: 94 °C for 2 minutes and 35 cycles of amplification (94 °C for 30 seconds, 56 °C for

45 seconds, and 72 °C for 2 minutes) followed by 1 final cycle of 5 minutes at 72 °C.

The PCR products were purified with the ExoStart kit. Sanger sequencing was performed using all the oligonucleotides described in Table 1 and analyzed with Seqman software (DNA Lasergene 12 package).

Microsatellite Typing

A panel of 4 short tandem repeat (STR) markers was used for genotyping the *C. parapsilosis* isolates. Three trinucleotide repeat markers and 1 hexanucleotide repeat marker described by Diab-Elschahawi [25] were independently amplified by PCR. Amplification reactions were performed in a final volume of 20 µL for markers 3A, 3B, and 6A, containing 1 ng of genomic DNA, 0.5 µM of amplification primers, 120 µM of dNTPS, 1.25 mM of $MgCl_2$, and 1 U of Amplitaq DNA (Applied Biosystems). The PCR conditions used were initial denaturalization for 5 minutes at 95 °C, followed by 30 cycles of 15 seconds of denaturalization at 95 °C, 1 minute of annealing at 62 °C, and 1 minute of extension at 72 °C. A final incubation of 7 minutes at 72 °C was included in the protocol. The PCR conditions were optimized for the "3C" marker. In this case, amplification reactions were performed in a final volume of 50 µL, containing 1 ng of DNA, 0.2-µM amplification primers, 0.05 mM of dNTPS, 0.3 mM of $MgCl_2$, and 1 U of Amplitaq DNA (Applied Biosystems). PCR conditions for the 3C marker were as follows: initial denaturalization for 5 minutes at 94 °C, followed by 35 cycles of 30 seconds of denaturalization at 94 °C, 45 seconds of annealing at 60 °C, and 1 minute of extension at 72 °C, followed by 5 minutes at 72 °C. Then, 10 µL of the amplification products was put on a PCR Plate 96 semiskirted (Eppendorf) and purified with AMpure XP (Beckman Coulter) using SPRI bead technology in an Eppendorf ep Motion 5075 (Eppendorf). Finally, a 1-µL aliquot of PCR product was added to 9 µL of formamide and to 1 µL of internal size marker GeneScan 500 ROX (Applied Biosystems). After denaturalization of the samples at 95 °C for 3 minutes and rapid cooling to 4 °C, they were run on an AB3730XL DNA analyzer (Applied Biosystems). Allele size analysis was performed with Peak scanner software (Applied Biosystems) according to the internal size standard GeneScan 500 ROX.

Similarities between genotypes were visualized by constructing a minimum spanning tree using InfoQuest FP, version 4.5 (Applied Maths, St.-Martens-Latem, Belgium), treating the data as categorical information.

Data Analysis and Statistics

MIC analysis was performed with SPSS software. For each year, the distribution of MICs was reported. We also calculated the geometric mean of the MIC values, the median, and the minimal and maximal values of the distributions.

RESULTS AND DISCUSSION

We collected all the isolates available at our laboratory (SMRL) and analyzed the evolution of the antifungal susceptibility pattern from 2000 to 2021. A total of 1315 isolates were studied. As shown in Table 1, resistance to fluconazole remained low (3%–7%) among the isolates from our collection until 2016. However, a dramatic change in this resistance rate among the isolates received at the Reference Laboratory was noted thereafter, being particularly notable from 2019 onwards. Throughout the latter period, the percentage of fluconazole resistance significantly increased (27% in 2019, around 60% in 2020 and 2021) (Table 2) as compared with previous years. This trend was also observed for voriconazole (Table 3). Before 2019, the voriconazole resistance rate was below 2%, but since 2020, the percentage of susceptible increased exposure (I, MIC = 0.25 mg/L) and resistant strains (MIC > 0.25 mg/L) increased up to around 60% among the strains received at the laboratory.

Regarding itraconazole and posaconazole, there was a slight trend toward higher MICs, but they were still categorized as susceptible (Tables 4 and 5). Only 3 isolates were fully resistant to fluconazole: voriconazole, itraconazole, and posaconazole. For isavuconazole, although there are no breakpoints to define resistant strains, an increase in the MICs among the isolates received since 2020 was found. The isavuconazole modal MIC rose from 0.016 mg/L before 2020 to 0.06 mg/L later on (Table 6), similar to what was observed for itraconazole and posaconazole. All the isolates were considered wild-type to the rest of the antifungals tested (AmB, flucytosine, caspofungin, micafungin, anidulafungin).

The presence of *ERG11* mutations in fluconazole non-susceptible isolates was investigated. The *ERG11* gene of 244 strains from 2019, 2020, and 2021, including S (n = 34), I (susceptible, increased exposure, n = 7), and R (n = 203) strains to FLC, was sequenced. The *ERG*11 gene was found to be wild-type in all the susceptible strains, and in 4.8% of the FNS isolates. Among the latter (n = 11), 1 strain was also susceptible increased exposure (I), and 6 were resistant to voriconazole. The remaining fluconazole-resistant isolates (n = 192, 94.6%) harbored the Y132F mutation, which has already been associated with FLC resistance in *C. parapsilosis* (Table 7). In addition, we found that 1 of the resistant isolates harbored the K143R mutation in the *ERG11* gene, which has been detected in azole-nonsusceptible strains causing monoclonal outbreaks in India [26], as well as in combination with the Y132F mutation [11]. This mutation has also been associated with pan-azole resistance in *C. tropicalis* [27]. Another strain harbored the G458S mutation, which has been related to azole resistance in *Candida parapsilosis* [4, 28]. Finally, many isolates harbored R398I (data not shown), but this mutation was also found in several susceptible isolates, which suggest that it is not related to FLC resistance.

Table 2. Distribution of the Percentage of MICs to Fluconazole of *C. parapsilosis* Strains Received at the SMRL Since 2000

	MIC, mg/L														
	Susceptible					I	Resistant								
Year	0.125	0.25	0.5	1	2	4	8	16	32	64	>64	No.	% S	% I	% R
2000	2	33	35	16	12	2	0	0	0	0	0	43	98	2	0
2001	1	30	53	12	1	3	0	0	0	0	0	74	97	3	0
2002	8	36	48	5	3	0	0	0	0	1	0	80	99	0	1
2003	11	40	41	5	1	0	1	1	0	0	0	115	98	0	2
2004	7	35	44	9	5	0	0	0	0	0	0	75	100	0	0
2005	18	41	32	6	1	0	0	0	1	0	0	82	99	0	1
2006	3	27	49	17	3	1	0	0	0	0	0	71	99	1	0
2007	3	24	56	8	6	0	1	0	0	0	1	71	97	0	3
2008	3	27	55	5	3	1	1	0	2	0	1	92	95	1	4
2009	0	12	78	4	0	0	4	1	0	0	0	73	95	0	5
2010	9	11	64	9	2	0	2	4	0	0	0	55	95	0	5
2011	5	14	59	14	0	0	9	0	0	0	0	22	91	0	9
2012	14	14	50	23	0	0	0	0	0	0	0	22	100	0	0
2013	0	14	57	21	0	0	7	0	0	0	0	14	93	0	7
2014	15	12	46	23	0	0	0	0	0	4	0	26	96	0	4
2015	0	2	61	37	0	0	0	0	0	0	0	46	100	0	0
2016	0	17	48	17	9	4	4	0	0	0	0	23	91	4	4
2017	9	22	39	4	4	4	4	9	4	0	0	23	78	4	17
2018	0	15	85	0	0	0	0	0	0	0	0	13	100	0	0
2019	13	7	7	13	7	7	0	26	7	0	13	15	47	7	46
2020	3	5	17	5	0	1	7	30	11	14	7	76	30	1	69
2021	3	13	12	1	4	3	10	36	11	5	1	204	33	3	64

The table includes the number of strains analyzed each year and the % of susceptible, susceptible increased exposure, and resistant isolates. Cells in orange: mode (most frequent value). In yellow: left and right values to the mode.

Abbreviations: I, susceptible increased exposure; MIC, minimum inhibitory concentration; R, resistant; S, susceptible; SMRL, Spanish Mycology Reference Laboratory.

Interestingly, in up to 35% of FNS strains, the Y132F substitution was found in heterozygosis. Thus, we analyzed if the triazole MIC distribution differed among homozygous or heterozygous strains. We observed that strains that harbored the Y132F mutation in homozygosis had higher MICs to fluconazole (geometric mean = 26.1 mg/L) compared with strains carrying the mutation in heterozygosis (geometric mean = 12.5). A similar situation was found for voriconazole (GM of heterozygous strains = 0.39 mg/L vs GM for homozygous strains = 0.5 mg/L). The Y132F substitution did not have a significant influence on susceptibility to isavuconazole, posaconazole, and itraconazole. Moreover, for these 3 antifungals, the Y132F mutation in homozygosis tended to result in lower GM than in heterozygous strains (Table 8).

To investigate if there was any genetic correlation between the FNS strains, we performed a microsatellite-based genotyping of 270 *C. parapsilosis* (from 2019, 2020, and 2021) isolates from 234 different patients and 8 environmental strains, including 81 susceptible, 6 susceptible increased exposure (I), and 183 resistant isolates (fluconazole categorization). The majority of the strains were isolated from blood (38.3% among FLC-S strains and 44.4% among FLC-R/I strains), skin (13.6% among FLC-S strains and 20.1% among FLC-R/I strains), and respiratory samples (9.9% among FLC-S strains and 11.64% among FLC-R/I strains). Among the susceptible isolates, we included strains from the same hospitals that had resistant strains, but also others not related to these outbreaks. Microsatellite genotyping identified 121 different genotypes. The relationship between the obtained genotypes is illustrated in Figures 1 and 2 and Supplementary Table 1.

As compared with the FNS isolates, genotypic variability was greater among fluconazole-susceptible strains, which could be attributed, in part, to the fact that most of the susceptible strains were recovered from unrelated cases.

Remarkably, in the case of contemporary resistant isolates, there was a markedly well-defined geographical distribution of genotypes. Genotype 10 was found to be the dominant cluster among strains of 2 hospitals from the area of Barcelona (Bellvitge Universitary Hospital and Vall d'Hebron Universitary Hospital). This cluster was noted for the first time in 3 isolates from 1 of these 2 centers by 2019 (Bellvitge Universitary Hospital), and was also detected in a third center of the metropolitan area of Barcelona that contributed with a single isolate sent to the reference laboratory the same year. These 2 former hospitals also shared the closely related genotype 12. Neither genotype 10 nor genotype 12 was found in

Table 3. Distribution of the Percentage of MIC to Voriconazole of *C. parapsilosis* Strains Received at the SMRL Since 2000

…	MIC, mg/L											…	…	…	…
…	Susceptible				I	Resistant						…	…	…	…
Year	0.016	0.031	0.06	0.125	0.25	0.5	1	2	4	8	>8	No.	% S	% I	% R
2000	79	14	7	0	0	0	0	0	0	0	0	42	100	0	0
2001	73	23	1	3	0	0	0	0	0	0	0	74	100	0	0
2002	85	14	0	0	0	1	0	0	0	0	0	80	99	0	1
2003	88	11	0	1	0	0	0	0	0	0	0	94	100	0	0
2004	96	4	0	0	0	0	0	0	0	0	0	75	100	0	0
2005	93	6	0	0	1	0	0	0	0	0	0	82	99	1	0
2006	94	4	1	0	0	0	0	0	0	0	0	71	100	0	0
2007	89	6	4	0	0	0	0	0	0	0	1	71	99	0	1
2008	90	4	0	2	0	1	1	1	0	0	0	92	97	0	3
2009	89	5	1	3	1	0	0	0	0	0	0	73	99	1	0
2010	85	4	4	5	2	0	0	0	0	0	0	55	98	2	0
2011	86	9	0	5	0	0	0	0	0	0	0	22	100	0	0
2012	77	18	5	0	0	0	0	0	0	0	0	22	100	0	0
2013	79	14	0	7	0	0	0	0	0	0	0	14	100	0	0
2014	69	23	4	0	0	0	4	0	0	0	0	26	96	0	4
2015	89	9	0	2	0	0	0	0	0	0	0	46	100	0	0
2016	70	13	13	4	0	0	0	0	0	0	0	23	100	0	0
2017	57	26	0	9	0	4	4	0	0	0	0	23	91	0	9
2018	87	0	0	0	0	13	0	0	0	0	0	15	87	0	13
2019	27	26	0	0	7	20	7	13	0	0	0	15	53	7	40
2020	24	7	0	1	13	22	32	1	0	0	0	76	32	13	55
2021	28	2	3	5	25	27	7	1	1	0	0	204	38	25	37

The table includes the number of strains analyzed each year and the % of susceptible, susceptible increased exposure, and resistant isolates. Cells in orange: mode (most frequent value). In yellow: left and right values to the mode.

Abbreviations: I, susceptible increased exposure; MIC, minimum inhibitory concentration; R, resistant; S, susceptible; SMRL, Spanish Mycology Reference Laboratory.

centers from other regions in Spain. Genotype 96 was found to be highly prevalent among isolates obtained from centers located in Madrid and Burgos (Castilla León Region). Genotype 67 was found in a hospital in the north of Spain (Hospital Marqués de Valdecilla, Santander, Cantabria), geographically distant from Madrid and Barcelona. Interestingly, genotype 67 was found in outbreaks from 2 distant hospitals, Son Espases Hospital (Balearic Islands) and Marqués de Valdecilla Hospital (Santander, Cantabria). The outbreak in the Balearic Islands has been previously described [29]. Additionally, fluconazole-susceptible strains isolated in the context of another nosocomial outbreak (Universitary Clinic Hospital from Valladolid) were received, displaying genotypes clearly different from the abovementioned and closely related to each other (genotypes 45–50). The geographical distribution of the genotypes of the resistant strains is shown in Figure 1.

A minimum spanning tree was built, showing that some genotypes have evolved by spontaneous changes in one of the microsatellite markers. The microsatellite analysis showed a distribution of clades that grouped by geographic origin, with resistant strains clustering together (Figure 2).

Our work shows a significant increase in the number of *C. parapsilosis* samples resistant to fluconazole and voriconazole received at the SMRL from several Spanish hospitals and arising in a relatively short period. These isolates seem to be part of outbreaks that have emerged almost simultaneously in distant cities and that can be attributed to clones that are shared almost exclusively among geographically close related centers. From these data, a generalized increment in fluconazole resistance among Spanish isolates of *C. parapsilosis* cannot be inferred as it is not mandatory to report all the infections caused by these species. All together, our data are in sharp contrast to what has being described in several former epidemiological studies carried out in Spain [6, 7, 30, 31], suggesting a new and worrisome change in the epidemiological incidence of FNS *C. parapsilosis* strains. Furthermore, our work also suggests that there has been a dispersion of several genotypes between different hospitals from the same and different regions. In particular, genotype 10 has disseminated through different hospitals and Cataluña, genotype 96 was first found in Madrid and Burgos, and genotype 67 was found in the Balearic Islands and Cantabria. Similar findings have recently been described from hospitals in Madrid [32]. When analyzing the sample isolation dates, our data suggest that the major clone that circulated in hospitals in Madrid disseminated to Burgos Hospital. Of interest is the case of the hospitals in the Balearic Islands and

Table 4. Distribution of the Percentage of MIC to Itraconazole of *C. parapsilosis* Strains Received at the SMRL Since 2000

	MIC, mg/L													
	Susceptible				Resistant									
Year	0.016	0.031	0.06	0.125	0.25	0.5	1	2	4	8	>8	No.	% S	% R
2000	58	30	9	2	0	0	0	0	0	0	0	43	100	0
2001	30	55	11	4	0	0	0	0	0	0	0	74	100	0
2002	25	56	18	0	1	0	0	0	0	0	0	80	99	1
2003	50	36	12	1	0	1	0	0	0	0	0	115	99	1
2004	61	31	7	1	0	0	0	0	0	0	0	75	100	0
2005	52	41	6	0	0	0	0	0	0	0	0	82	100	0
2006	73	20	6	1	0	0	0	0	0	0	0	71	100	0
2007	63	25	8	1	0	0	0	0	0	0	1	71	99	1
2008	52	40	4	0	2	1	0	0	0	0	0	92	97	3
2009	78	21	0	1	0	0	0	0	0	0	0	73	100	0
2010	95	0	2	2	2	0	0	0	0	0	0	55	98	2
2011	86	9	5	0	0	0	0	0	0	0	0	22	100	0
2012	95	5	0	0	0	0	0	0	0	0	0	22	100	0
2013	71	29	0	0	0	0	0	0	0	0	0	14	100	0
2014	65	23	4	0	4	4	0	0	0	0	0	26	92	8
2015	72	28	0	0	0	0	0	0	0	0	0	46	100	0
2016	35	22	30	13	0	0	0	0	0	0	0	23	100	0
2017	26	35	22	9	9	0	0	0	0	0	0	23	91	9
2018	7	20	67	7	0	0	0	0	0	0	0	15	100	0
2019	7	13	33	40	7	0	0	0	0	0	0	15	93	7
2020	15	20	40	23	2	0	0	0	0	0	0	66	98	2
2021	23	28	22	20	6	1	1	0	0	0	0	204	89	11

Cells in orange: mode (most frequent value). In yellow: left and right values to the mode.

Abbreviations: MIC, minimum inhibitory concentration; SMRL, Spanish Mycology Reference Laboratory.

Santander (Cantabria), which are more distant than the rest of the hospitals described in this work. In this case, since the outbreak in Son Espases Hospital occurred in 2015 [29], we hypothesized that this clone was disseminated to Cantabria.

Recent emergence of FNS isolates in *C. parapsilosis* has been described in other countries in the literature [11–20], so our data support that the increase of azole resistance in *C. parapsilosis* might be a global problem. In this study, the majority of resistant isolates harbored the Y132F mutation, which has been largely associated in the literature with the appearance of clonal outbreaks. However, we also detected a few isolates that did not have this mutation. For this reason, further studies should be performed to describe all the resistance mechanisms circulating among Spanish hospitals.

At the moment, the reasons for the increase in the incidence of azole-resistant *C. parapsilosis* strains in Spain are unknown, but we hypothesized that this phenomenon may be related to the negative impact that the COVID-19 pandemic has had in Spanish hospitals for several reasons. There seems to be a clear temporal correlation between the increase in the number of resistant isolates received at the reference laboratory and the clinical impact of the pandemic. The COVID-19 pandemic has resulted in a severe overcrowding of hospitals and, in particular, of intensive care units, along with the necessity of recruiting large numbers of health care professionals that were not properly trained in infection control measures. Moreover, during the pandemic, there were changes in personal protective equipment protocols, and the same gloves could have been used between patients [33, 34]. This might have increased the risk of cross-transmission between patients and caused hospital outbreaks. Furthermore, during the pandemic, there has been a significant transfer of patients and health care professionals between different hospitals, which might have contributed to the dispersion of resistant clones between clinical tertiary centers. A similar situation has been described in multicenter studies in India [26], which highlights the ability of FNS isolates to spread and colonize hospital environments. Interestingly, some of the analyzed strains in this work in 1 of the hospitals (Bellvitge Universitary Hospital) were isolated from an environmental origin in the hospital, and we found that the most prevalent genotype obtained from patients in this hospital (genotype 10) was also present in different hospital locations. This correlation suggests that *C. parapsilosis* clones mighospital surfaces, which increases the risk of recirculating among patients and, in parallel, increases the risk of invasive infections among the most fragile individuals. Previous studies, in fact, have shown not only an increased incidence of *C. parapsilosis* infections in COVID-19 patients [35], but also other fungal

Table 5. Distribution of the Percentage of MIC to Posaconazole of *C. parapsilosis* Strains Received at the SMRL Since 2000

	MIC, mg/L													
	Susceptible			Resistant										
Year	0.016	0.031	0.06	0.125	0.25	0.5	1	2	4	8	>8	No.	% S	% R
2001	55	45	0	0	0	0	0	0	0	0	0	11	100	0
2002	65	31	4	0	0	0	0	0	0	0	0	80	100	0
2003	80	19	1	0	0	0	0	0	0	0	0	94	100	0
2004	83	17	0	0	0	0	0	0	0	0	0	75	100	0
2005	65	33	2	0	0	0	0	0	0	0	0	82	100	0
2006	87	13	0	0	0	0	0	0	0	0	0	70	100	0
2007	87	11	0	0	0	0	0	0	1	0	0	71	99	1
2008	70	26	1	1	1	1	0	0	0	0	0	92	97	3
2009	81	19	0	0	0	0	0	0	0	0	0	73	100	0
2010	74	20	2	2	2	0	0	0	0	0	0	54	96	4
2011	95	5	0	0	0	0	0	0	0	0	0	22	100	0
2012	91	9	0	0	0	0	0	0	0	0	0	22	100	0
2013	93	7	0	0	0	0	0	0	0	0	0	14	100	0
2014	92	4	4	0	0	0	0	0	0	0	0	26	100	0
2015	78	20	2	0	0	0	0	0	0	0	0	46	100	0
2016	22	52	22	4	0	0	0	0	0	0	0	23	96	4
2017	26	57	13	4	0	0	0	0	0	0	0	23	96	4
2018	13	47	33	7	0	0	0	0	0	0	0	15	93	7
2019	13	41	33	13	0	0	0	0	0	0	0	15	87	13
2020	33	45	20	2	0	0	0	0	0	0	0	76	98	2
2021	23	38	20	12	4	2	1	0	0	0	0	204	81	19

Cells in orange: mode (most frequent value). In yellow: left and right values to the mode.

Abbreviations: MIC, minimum inhibitory concentration; SMRL, Spanish Mycology Reference Laboratory.

Table 6. Distribution of the Percentage of MIC to Isavuconazole of *C. parapsilosis* Strains Received at the SMRL Since 2016

	MIC, mg/L											
…												…
Year	0.016	0.031	0.06	0.125	0.25	0.5	1	2	4	8	>8	No.
2016	86	14	0	0	0	0	0	0	0	0	0	7
2017	75	15	5	0	5	0	0	0	0	0	0	20
2018	87	7	7	0	0	0	0	0	0	0	0	15
2019	53	13	7	13	7	7	0	0	0	0	0	15
2020	39	14	39	8	0	0	0	0	0	0	0	72
2021	38	23	29	7	2	1	1	0	0	0	0	196

Cells in orange: mode (most frequent value). In yellow: left and right values to the mode.

Abbreviations: MIC, minimum inhibitory concentration; SMRL, Spanish Mycology Reference Laboratory.

Table 7. Mutations in the *ERG11* Gene Found in Susceptible, Susceptible Increased Exposure, and Resistant Strains to Fluconazole

ERG11 Mutation	FLC Susceptible	FLC Susceptible Increased Exposure			FLC Resistant		
…	VOR_S	VOR_S	VOR_I	VOR_R	VOR_S	VOR_I	VOR_R
WT	34	1	1	0	5	1	6
Y132F_HET	0	3	1	0	1	17	38
Y132F_HOMO	0	0	0	1	5	39	88

For each category, we also include the susceptibility profile (S/I/R) for voriconazole.

Abbreviations: HET, heterozygous; HOMO, homozygous; I, susceptible increased exposure; MIC, minimum inhibitory concentration; R, resistant; S, susceptible; SMRL, Spanish Mycology Reference Laboratory.

Table 8. Susceptibility Profile of WT or Mutant Strains Harboring the Y132F in Homozygosity or Heterozygosity

… Antifungal	… *ERG11* Mutation	… No.	Antifungal Susceptibility, mg/L			
			Median	Geometric Mean	Minimal	Maximal
Fluconazole	WT	43	0.5	0.78	0.125	>64
	Y132F_HET	60	16	12.7	4	32
	Y132F_HOM	120	16	25	8	>64
Voriconazole	WT	43	0.031	0.032	0.016	4
	Y132F_HET	60	0.5	0.4	0.125	1
	Y132F_HOM	120	0.5	0.5	0.06	2
Itraconazole	WT	43	0.03	0.033	0.016	1
	Y132F_HET	60	0.125	0.10	0.031	0.25
	Y132F_HOM	120	0.06	0.05	0.016	0.25
Posaconazole	WT	43	0.03	0.029	0.016	0.25
	Y132F_HET	60	0.06	0.065	0.016	0.5
	Y132F_HOM	120	0.031	0.035	0.016	0.25
Isavuconazole	WT	43	0.016	0.021	0.016	1
	Y132F_HET	60	0.06	0.07	0.031	0.125
	Y132F_HOM	120	0.031	0.037	0.016	0.5

Abbreviation: WT, wild-type.

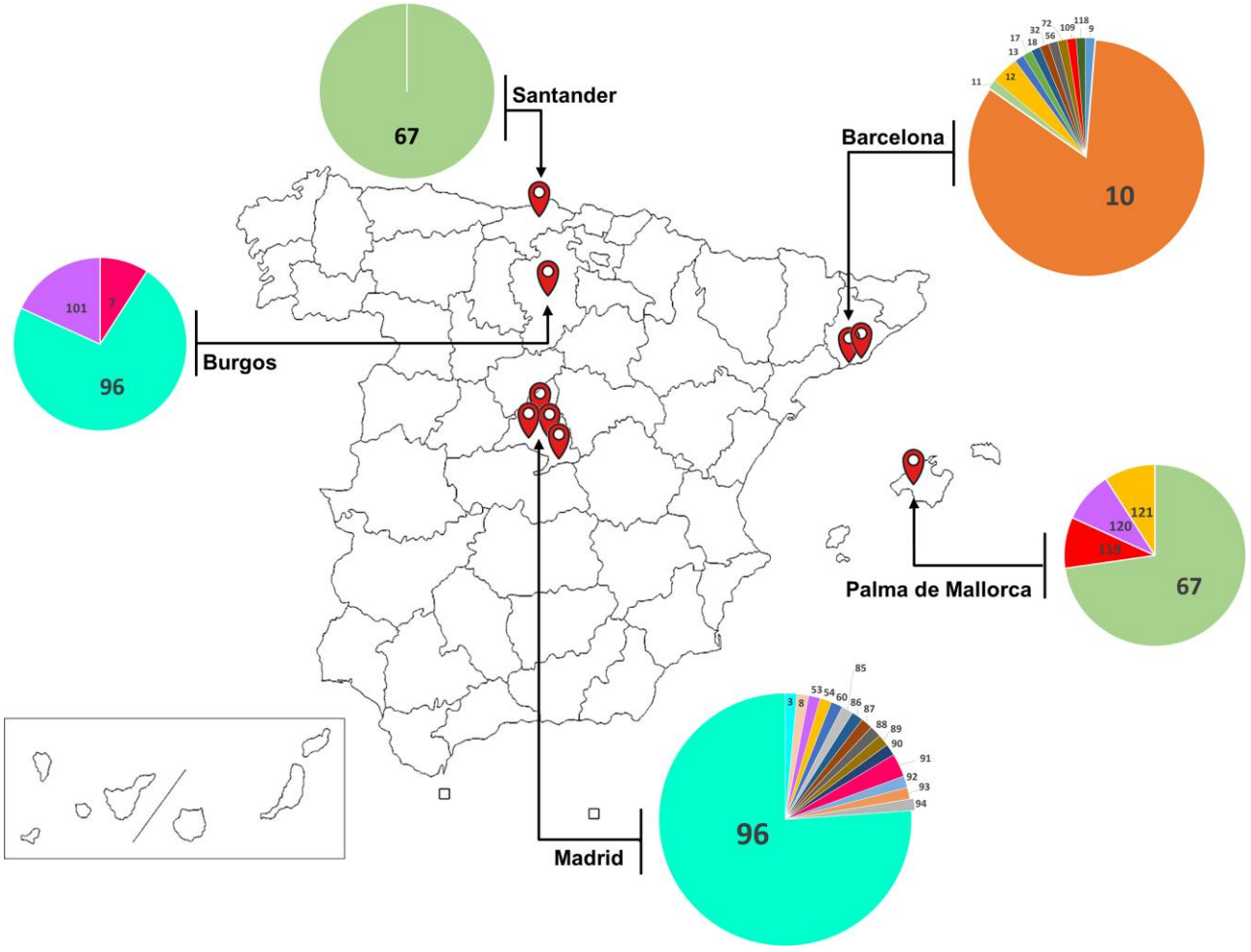

Figure 1. Geographical distribution of the different genotypes of FLC-resistant isolates. The pie charts denote the distribution of the different genotypes in different tertiary hospitals from different metropolitan areas in Spain. Abbreviation: FLC, fluconazole. Template of the map of Spain was obtained from a free repository (https://es.m.wikipedia.org/wiki/Archivo:Provinces_of_Spain_%28Blank_map%29.png) and its use and modification is allowed according to the GNU Free Documentation License, version 1.2.

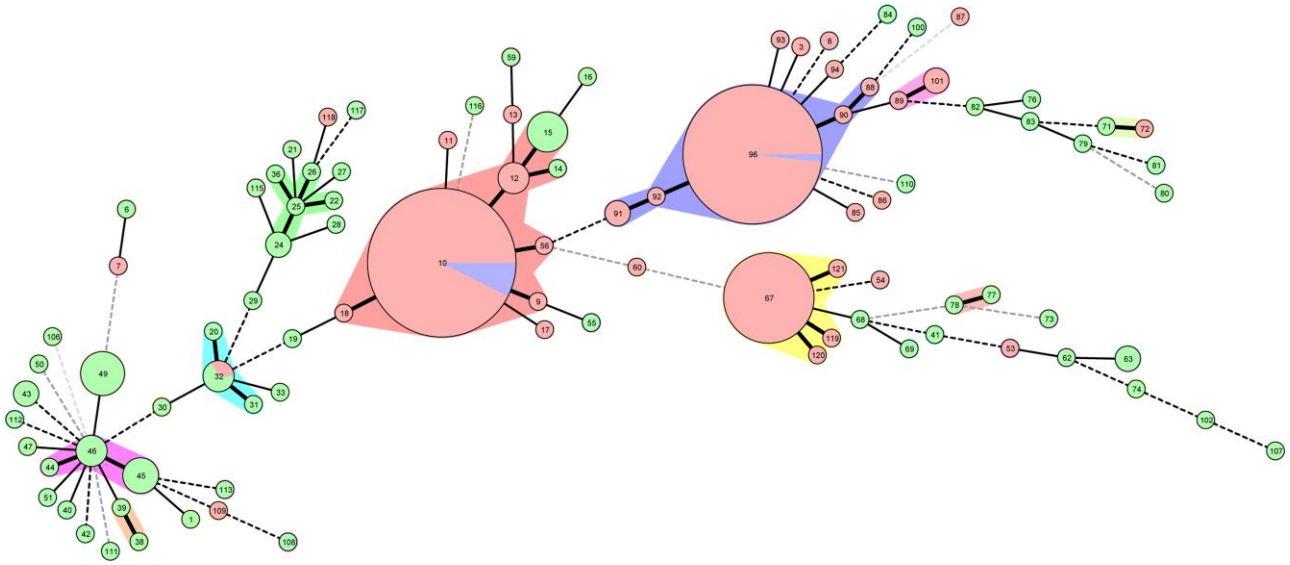

Figure 2. Minimal spanning tree showing the genetic proximity of susceptible and FLC-resistant isolates from *Candida parapsilosis*. The numbers denote the genotype identified in each group. Straight bold lines denote groups that only differentiate in 1 marker. These groups are highlighted with the color shadows in the background. Orange: resistant strains; green: susceptible strains; blue: susceptible increased exposure (*I*) strains. For the origin and a description of the strains in each genotype, see Supplementary Table 1. Abbreviation: FLC, fluconazole.

Table 9. General Fluconazole and Voriconazole Use (Expressed and Defined Daily Doses per 100 Patients Days) in Several Hospitals From Different Geographical Regions From 2018 to 2021

…	…	2018	2019	2020	2021
Vall d'Hebron University Hospital[a]	Fluconazole	3.94	2.68	1.98	2.43
	Voriconazole	0.96	0.56	0.76	1.08
12 de Octubre Hospital	Fluconazole	2.21	2.31	3.05	2.56
	Voriconazole	0.26	0.4	0.34	0.32
Marqués de Valdecilla University Hospital	Fluconazole	2.16	2.11	2.24	1.94
	Voriconazole	1.12	1.29	1.99	1.63
Burgos University Hospital	Fluconazole	3.73	3.98	4.26	2.74
	Voriconazole	0.39	0.13	0.22	0.55
Móstoles University Hospital	Fluconazole	2.41	2.24	3.30	3.37
	Voriconazole	0.75	0.73	0.69	0.64
Puerta de Hierro University Hospital	Fluconazole	3.75	2.83	4.5	4.12
	Voriconazole	0.48	0.53	0.47	0.47
Bellvitge University Hospital	Fluconazole	1.61	1.46	1.89	1.53
	Voriconazole	0.38	0.3	0.55	0.71

[a]Data from use in the intensive care unit.

diseases, such as COVID-associated pulmonary aspergillosis (CAPA) [36–38], mucormycosis [39–41], and *Candida* infections [42, 43] (see reviews in [44, 45]).

The impact of the COVID-19 pandemic and clinical management of COVID patients does not fully explain why there has been a selection of azole-resistant strains and why these genetically different resistant strains have emerged almost simultaneously in distant places across Spain. An increase in the use of antimicrobials has been reported since the appearance of the COVID-19 pandemic in some geographical regions [46]. Among azoles, an increase in the use of echinocandins and voriconazole has been reported [46], which might have favored the selection of fluconazole and voriconazole-resistant *C. parapsilosis*. To validate this hypothesis, we were able to obtain data on fluconazole and voriconazole use from some of the hospitals, and, as shown in Table 9, there was not a significant increase in the use of fluconazole. A similar trend was found for voriconazole use, although 2 hospitals (Burgos Universitary Hospital and Bellvitge Universitary Hospital) reported an increase of around 2-fold in the use of this last antifungal. These data suggest that the increase in the incidence of FNS strains from *C. parapsilosis* has not been mainly driven by the selective pressure of the antifungal use. In agreement, no correlation between previous azole treatment and infection by FNS *C. parapsilosis* strains has been found in the outbreaks from Son Espases Universitary Hospital (Balearic Islands) and Puerta de Hierro Universitary Hospital (Madrid) [29, 47].

Another possibility is that resistance to azoles affects virulence traits. In this sense, it has been described that *C. parapsilosis* strains harboring the Y132F mutation in *ERG11* have reduced ability to form biofilms [11], which raises the hypothesis that these strains have a greater ability to spread and disseminate. Furthermore, several studies have associated the incidence of resistant strains with higher mortality of the patients [11, 28], which warrants further studies on the virulence of FLC-nonsusceptible *C. parapsilosis* strains. In our case, the

clinical management of the patients might have contributed to the selection of preexisting resistant clones circulating in the hospitals before the COVID-19 pandemic [47]. In our case, this idea is supported by the fact that we identified that some of the resistant clones were already detected in samples from 2019 (ie, Bellvitge Hospital) and also in some strains present in our collection from 2019 from the same geographical region. For this reason, future research to investigate the genetic proximity of the resistant isolates is needed, and to compare them not only between different hospitals but also with isolates described in different countries.

Despite the epidemiological limitations and interpretations of our work, we believe that the data presented herein are an indicator of an emerging clinical problem, that is, the selection of azole resistance in *C. parapsilosis* during the COVID-19 pandemic. In particular, we report the appearance of a significant increase in the resistance rate to fluconazole and voriconazole simultaneously in multiple hospitals in Spain. This increase has a temporal correlation with the COVID-19 pandemic, suggesting that the increased incidence of FNS *C. parapsilosis* strains is a consequence of the impact of the pandemic. We also present data that indicate that there has been a dissemination of some genotypes between hospitals, not only from the same cities but from different geographical regions, and despite the clonal diversity documented, only a few of them dominated across centers. We would like to note that the increase in FLC-resistant isolates in tertiary hospitals in Spain is agreement with the worldwide context, where an increasing number of outbreaks caused by FNS *C. parapsilosis* strains are being reported. Our work highlights the importance of national surveillance programs carried out by reference laboratories to detect epidemiological changes and to characterize outbreaks, especially those that involve the selection of microbial-resistant isolates. We encourage the clinical community to investigate the presence of these clones in the hospital environment, to make an effort to perform susceptibility testing in strains of noninvasive origins (colonization, isolated from hospital surfaces, etc.), and to design specific measures to prevent the expansion of the associated resistance mechanisms.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Acknowledgments

Financial support. O.Z. was funded by grants SAF2017–86912-R and PID2020–114546RB-I00 from the Spanish Ministry for Science and Innovation. This work was also funded by the National Centre for Microbiology (Instituto de Salud Carlos III) through the Surveillance Program of Antifungal Resistance and the Center for Biomedical Research in Network of Infectious Diseases CIBERINFECTCB21/13/00105 (O.Z. and L.A.F.), CIBERINFEC-CB21/13/00009 (M.P.-A.), CIBERES-CB06/06/0037 (C.A.-T.), and CIBERES-CB06/06/0058 (J.G). L.A.-F. was supported by Fondo de Investigación Sanitaria (MPY 117/18 and MPY 305/20). We thank Dr. David Campany Herrero (Vall d'Hebron Hospital), Noelia Garrido Peño (Móstoles Hospital), David Gómez Gómez y Aitziber Illaro Uranga (Marqués de Valdecilla Hospital), María Ángeles Machín Morón (Burgos Hospital), Jose Manuel Caro Teller (Doce de Octubre Hospital), Marina Calvo (Puerta de Hierro Hospital), and Ariadna Padulles (Bellvitge Hospital) for providing the data on antifungal consumption from their hospitals. We also thank Ángel Zaballos and Pilar Jiménez from the Genomics Core Facility from Instituto de Salud Carlos III for their technical help with the microsatellite analysis technique.

Potential conflicts of interest. All authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

Patient consent. This study does not include factors necessitating patient consent.

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