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MECHANISMS OF RESISTANCE

First Report of Candidemia Clonal Outbreak Caused by Emerging Fluconazole-Resistant *Candida parapsilosis* Isolates Harboring Y132F and/or Y132F+K143R in Turkey

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ABSTRACT Clonal outbreaks of fluconazole-resistant (FLZR) *Candida parapsilosis* isolates have been reported in several countries. Despite its being the second leading cause of candidemia, the azole resistance mechanisms and the clonal expansion of FLZR *C. parapsilosis* blood isolates have not been reported in Turkey. In this study, we consecutively collected *C. parapsilosis* blood isolates ($n = 225$) from the fifth largest hospital in Turkey (2007 to 2019), assessed their azole susceptibility pattern using CLSI M27-A3/S4, and sequenced *ERG11* for all and *MRR1*, *TAC1*, and *UPC2* for a selected number of *C. parapsilosis* isolates. The typing resolution of two widely used techniques, amplified fragment length polymorphism typing (AFLP) and microsatellite typing (MST), and the biofilm production of FLZR isolates with and without Y132F were compared. Approximately 27% of isolates were FLZR (60/225), among which 90% (54/60) harbored known mutations in Erg11, including Y132F (24/60) and Y132F+K143R (19/60). Several mutations specific to FLZR isolates were found in *MRR1*, *TAC1*, and *UPC2*. AFLP grouped isolates into two clusters, while MST revealed several clusters. The majority of Y132F/Y132F+K143R isolates grouped in clonal clusters, which significantly expanded throughout 2007 to 2019 in neonatal wards. *Candida parapsilosis* isolates carrying Y132F were associated with significantly higher mortality and less biofilm production than other FLZR isolates. Collectively, we documented the first outbreak of FLZR *C. parapsilosis* blood isolates in Turkey. The *MRR1*, *TAC1*, and *UPC2* mutations exclusively found in FLZR isolates establishes a basis for future studies, which will potentially broaden our knowledge of FLZR mechanisms in *C. parapsilosis*. MST should be a preferred method for clonal analysis of *C. parapsilosis* isolates in outbreak scenarios.

KEYWORDS *Candida*, *Candida parapsilosis*, drug resistance mechanisms, fluconazole

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Bloodstream infections due to *Candida* species, i.e., candidemia, are associated with high morbidity and mortality, resulting in significant health care costs of $1.4 billion in the United States annually (1). *Candida parapsilosis* inhabits the gastrointestinal tract of 35% of healthy individuals (2) and ranks as the first to third cause of candidemia depending on geography, patients' underlying condition, and age (3). The ability of *C. parapsilosis* to produce tenacious biofilms accounts for its persistence in clinical settings and poses the risk of future clonal outbreaks (3). The sibling species of *C. parapsilosis*, *Candida orthopsilosis* and *Candida metapsilosis*, have also been implicated in candidemia (4). *Candida parapsilosis* blood isolates were once thought to be universally fluconazole (FLZ) susceptible (FLZS), but a recent candidemia study conducted in South Africa indicates that over half of *C. parapsilosis* isolates are FLZ resistant (FLZR), and 44% of the latter are cross-resistant to voriconazole (VRZ) (5). Other studies performed in India (6), South Korea (7), Kuwait, Brazil (8), and the United States (9, 10) and a recent global study (11) confirmed the emergence of FLZR *C. parapsilosis*. Given that FLZ is the main antifungal drug used in developing countries (5, 12), the emergence of FLZR isolates undermines the efficacy of FLZ in the treatment of candidemia. Prolonged previous exposure to FLZ in clinical settings is believed to be a factor related to FLZ resistance (13), which is underlain by specific mutations in the ergosterol biosynthesis gene, *ERG11*, yielding amino acid substitutions such as Y132F and K143R, which alter the 3D conformation of Erg11 and reduce its affinity for FLZ (14). Moreover, gain-of-function (GOF) mutations in *MRR1*, *TAC1*, and *UPC2* genes that cause overexpression of efflux pumps (Cdr1 and Mdr1) and Erg11 are also known to contribute to azole resistance in *Candida* species (14).

Candida parapsilosis infections may be spread by health care workers (3), and isolates from outbreaks can be more virulent than sporadic isolates (15). As *C. parapsilosis* is among the most genetically homogenous *Candida* species (3), the use of highly resolutive typing techniques is important to differentiate outbreak from nonoutbreak isolates. Amplified fragment length polymorphism typing (AFLP) (16) and microsatellite typing (MST) (6) have been used to explore the genotypic diversity of clinical *C. parapsilosis* isolates, but there is no study comparing the performance of the two techniques.

In this single-center study, we investigated the nature of an unusually high prevalence of *C. parapsilosis* blood isolates collected over 13 years (2007 to 2019) in Ege University Hospital, Izmir, Turkey, in order to track the evolution of *C. parapsilosis* azole resistance over time. We assessed the genetic relatedness of *C. parapsilosis* isolates using AFLP and MST and explored a recent hypothesis that FLZR *C. parapsilosis* isolates carrying the Y132F mutation may have a higher propensity to persist in clinical settings (15).

RESULTS

Patients' clinical profiles. In total, 225 *C. parapsilosis* and 2 *C. orthopsilosis* isolates were recovered from 223 patients; 54.4% ($n = 123$) of the patients were men and 40.3% ($n = 91$) were women (no data for 12 cases). Children (<18 years old) ($n = 95$; 42%) and adults (≥18 years old) ($n = 107$; 47.3%) almost equally developed candidemia due to *C. parapsilosis* (no data for 24 cases). Prophylactic treatment with antifungals was not performed for 133 patients (58.8%); when it was performed, the most frequent choice was FLZ ($n = 40$; 17.7%) followed by VRZ ($n = 9$; 4%) (no data for 45 patients; 19.5%). Since fluconazole treatment, both prophylactic and targeted, was heavily used from 2015 onward, our analysis focused on microbiological changes observed before and after 2014. Accordingly, the use of FLZ for prophylaxis increased 2.4-fold ($n = 25$ [26.3%] versus 15 [11.7%]; 95% confidence interval [CI], 1.328 to 5.451; odds ratio [OR] = 2.690; $P = 0.006$) and that of VRZ decreased 6-fold ($n = 1$ [1.1%] versus 8 [6.2%]; 95% CI, 0.02 to 1.298; OR = 0.160; $P = 0.086$) in the second phase (2015 to 2019) compared to the first phase (2007 to 2014). Considering the targeted treatment, the majority of the patients ($n = 137$; 60.6%) received a single antifungal drug, whereas 8% of patients were treated by a combination of two drugs ($n = 18$; 8%), and 12.4%

($n = 25$) did not receive any antifungals (no data for 43 cases). FLZ was the most widely used single drug ($n = 51$; 22.6%), followed by caspofungin ($n = 26$; 11.5%), amphotericin B AMB ($n = 25$; 11.1%), anidulafungin ($n = 16$; 7.1%), micafungin ($n = 11$; 4.9%), and VRZ ($n = 8$; 3.5%). The use of FLZ was tripled ($n = 35$ [36.8%] versus 16 [12.5%]) and that of echinocandins doubled ($n = 24$ [25.2%] versus 16 [12.2%]) in the second phase compared to the first phase. The overall crude mortality rate was 38.1% ($n = 86$; no data for 40 patients). As we did not have mortality data for 32.1% of patients ($n = 42$) in the first phase, we could not compare the rates between the two phases. In terms of treatment, patients who received AMB formulations had the highest mortality ($n = 15$; 60%), and those treated with echinocandins ($n = 27$; 50.9%) and FLZ ($n = 21$; 42%) had similar mortality. The annual rate of *C. parapsilosis* isolation showed a sinusoidal trend, with peaks in 2012 and 2018 (see Fig. S1 in the supplemental material).

Antifungal susceptibility. In total, 26.4% of the isolates were FLZR ($n = 60$); among them, 31.6% ($n = 19$) were cross-resistant to VRZ (Table S3). The rate of FLZR isolates doubled in the second phase ($n = 34$; 35.4%) compared to the first phase ($n = 26$; 19.6%) (Table 1). The number of VRZR isolates was comparable between the first and second phases ($n = 11$ [8.3%] versus 8 [9.4%]), whereas that of isolates with intermediate susceptibility to VRZ tripled in the second phase ($n = 33$ [34.7%] versus 10 [7.5%]). Overall, the frequency of FLZR isolates increased and was the highest in 2018, constituting almost half of the total number of isolates collected in that year (Table 1 and Fig. S2).

Sequencing of genes implicated in azole resistance. Most FLZR isolates (90%; $n = 54$) carried Erg11 mutations known to cause FLZ resistance in *C. parapsilosis* (6) or *C. orthopsilosis* (17); among them, isolates carrying Y132F alone or in combination with other mutations constituted 90% ($n = 54$; 90%) (Table 1). Among FLZ resistance-related mutations, Y132F was the most prevalent ($n = 24$; 44.4%) followed by Y132F+K143R ($n = 19$; 35.1%), G458S ($n = 6$; 11.1%) (G458S alone, $n = 4$; G458S+T519A, $n = 1$; Q250K+R398I+G458S, $n = 1$), Y132F+G307A ($n = 3$; 5.5%), and K143R ($n = 2$; 3.7%) (Table 1; also Table S3). Heterozygosity was noted in five and six isolates carrying Y132F and Y132F+K143R, respectively, and the rest of the isolates were homozygotic for the mutations observed. Isolates with Y132F alone tripled in the second phase ($n = 18$ [75%] versus 6 [25%]), whereas those with Y132F+K143R were more prevalent in the first phase ($n = 12$ [63.1%] versus 7 [36.8%]) (Fig. 1). Isolates carrying Y132F+G307A ($n = 3$), G458S ($n = 4$), and G458S+T519A ($n = 1$) were detected only in the second phase, whereas those harboring K143R ($n = 2$) and G458S+R398I+Q250K ($n = 1$) were detected only in the first phase (Table 1; Table S3). Interestingly, 52% and 50% of FLZR isolates with or without *ERG11* mutations, respectively, were recovered from pediatric wards. Thus, all isolates carrying K143R ($n = 2$), Y132F+G307A ($n = 3$), and G458S ($n = 6$), 63.1% of Y132F+K143R isolates ($n = 12$), and 20.8% of isolates carrying only Y132F ($n = 5$) were detected in pediatric wards. Most VRZR isolates ($n = 15$; 78.94%) carried Y132F+K143R mutations, followed by Y132F ($n = 3$; 15.7%) and G458S ($n = 1$; 5.2%) (Table 1; also Table S3). Of note, most patients infected with isolates carrying Y132F ($n = 17$) died despite treatment with various antifungals; this rate was significantly higher (95% CI, OR = 6.8; $P = 0.005$) than that for patients infected with Y132F+K143R isolates (26.3%; $n = 5$). Among FLZR isolates carrying Erg11 mutations, 48.1% ($n = 26$), 16.6% ($n = 9$), and 11.1% ($n = 6$) also harbored nonsynonymous mutations in *UPC2*, *TAC1*, and *MRR1*, respectively (Table 1; also Table S3). All Mrr1 mutations were found in FLZR isolates, except for one FLZS isolate carrying K606E in Mrr1. Furthermore, P45H, Q371H, and Q372H in Upc2 and A21V, Q965K+M966V, P150H, D603V+P803L, and S760R+A761G in Tac1 were found exclusively in FLZR isolates (Table 1; also Table S3). Among FLZ- and VRZ-cross-resistant isolates, one (5.2%) harbored a unique Q965K+M966V ($n = 1$; 5.2%) mutation in Tac1, but none had mutations in Mrr1 or Upc2 specific to this phenotype.

Genotyping *C. parapsilosis* isolates. Compared to MST, AFLP revealed a higher degree of genetic similarity among the 225 isolates, and all isolates were clustered in

TABLE 1 Isolates ($n = 91$) used for sequencing of genes implicated in azole resistance[a]

Isolate group and cluster (*n*)	Ward (*n*)	No. of isolates	MIC (mg/liter)		Mutation (*n*) in:				Yr (*n*)
			FLZ	VRZ	Erg11p	Mrr1p	Tac1p	Upc2p	
Fluconazole-resistant isolates containing Y132F mutation									
A (18)	Pediatric surgery (5)	19	32 or >32	0.06–4	Y132F+K143R	G472V (1)	A21V (1)	L38I (3)	2011 (2)
C (1)	Pediatric (4)					L926* (1)	Q965K+M966V (1)	L38I+A793S (3)	2012 (3)
	Cardiac surgery (3)							P45H (1)	2013 (3)
	Pediatric oncology (2)							A793S (2)	2014 (4)
	Anesthesiology (2)								2016 (2)
	Pediatric ICU (1)								2017 (1)
	Infectious diseases (1)								2018 (2)
	Neurology (1)								2019 (2)
A (2)	Pediatric (2)	3	16–>32	0.5–2	Y132F+G307A			L38I+Q371H (1)	2016 (1)
C (1)	Pediatric surgery (1)								2018 (2)
A (4)	Anesthesiology (7)	24	8–32	0.06–2	Y132F	G427V (1)	N7Y+L578M (1)	E7* (1)	2011 (1)
B (11)	Pediatric (3)					Q1027R (2)	A21V (1)	L38I (1)	2012 (3)
C (1)	Thoracic surgery (3)						P150H (1)	L38I+A793S (2)	2013 (1)
D (4)	Chest diseases (3)						D603V+P803L (1)	Q372H (1)	2014 (1)
E (3)	General surgery (2)						L578M (1)	P45H (1)	2017 (3)
F (1)	Neurosurgery (2)							P45H+A793S (1)	2018 (12)
	Pediatric oncology (1)							A793S (5)	2019 (3)
	Pediatric Surgery (1)								
	Emergency (1)								
	Neurology (1)								
Isolates with other mutations causing fluconazole resistance (K143R and G458S)									
D (1)	Pediatric (2)	2	16–>32	0.5	K143R		A21V (1)	L38I (1)	2011 (1)
E (1)								A793S (1)	2013 (1)
D (3)	Pediatric surgery (2)	4	16–>32	0.5–1	G458S			Q371H (1)	2016 (1)
E (1)	Pediatric ICU (1)								2017 (1)
	Pediatric ICU (1)								2018 (2)
C (1)	Pediatric oncology (1)	1	16	0.25	Q250K+R398I+G458S	P295L+Q1074* (1)	L390I (1)		2014 (1)
F (1)	Pediatric surgery (1)	1	16	0.5	G458S+T519A			A793S (1)	2019 (1)
Other fluconazole-resistant isolates without Erg11 mutations causing fluconazole resistance									
A (1)	Pediatric surgery (3)	6	8–>32	0.03-0.5	R398I (3)	L419F (1)	G490R+S760R+A761G (1)	G342S (1)	2013 (4)
C (3)	Cardiac surgery (1)								2014 (1)
E (2)	Anesthesiology (1)								2017 (1)
	General surgery + organ transplant (1)								

(Continued on next page)

TABLE 1 (Continued)

Isolate group and cluster (*n*)	Ward (*n*)	No. of isolates	MIC (mg/liter)		Mutation (*n*) in:				Yr (*n*)
			FLZ	VRZ	Erg11p	Mrr1p	Tac1p	Upc2p	
Fluconazole-susceptible (dose dependent) isolates									
B (1)	Chest diseases (2)	3	4	0.03–0.25	R398I (2)		L578M (1)	A793S (1)	2013 (1)
C (2)	Pediatric (1)								2014 (2)
Fluconazole-susceptible isolates									
	Anesthesiology (5)	28	0.06–2	0.0156–0.06	R398I (13)	K606E (1)	N7I (2)	L38I (1)	2009 (1)
	Chest diseases (5)						N7Y+A352V (1)	L38I+A793S (2)	2012 (7)
	Internal medicine (3)						F186I (2)	Q348P (1)	2013 (10)
	Pediatric surgery (3)						E312D (1)	P201S (1)	2014 (3)
	Pediatric (3)						L390I (1)	S577* (1)	2018 (2)
	Gastroenterology (2)						G490R (1)	A793S (5)	2019 (5)
	Pediatric oncology (2)						L574F (1)		
	General surgery (2)						L578M (1)		
	Cardiac surgery (2)						L578M+N602Y (1)		
	Infectious diseases (1)								

[a]*, stop codon.

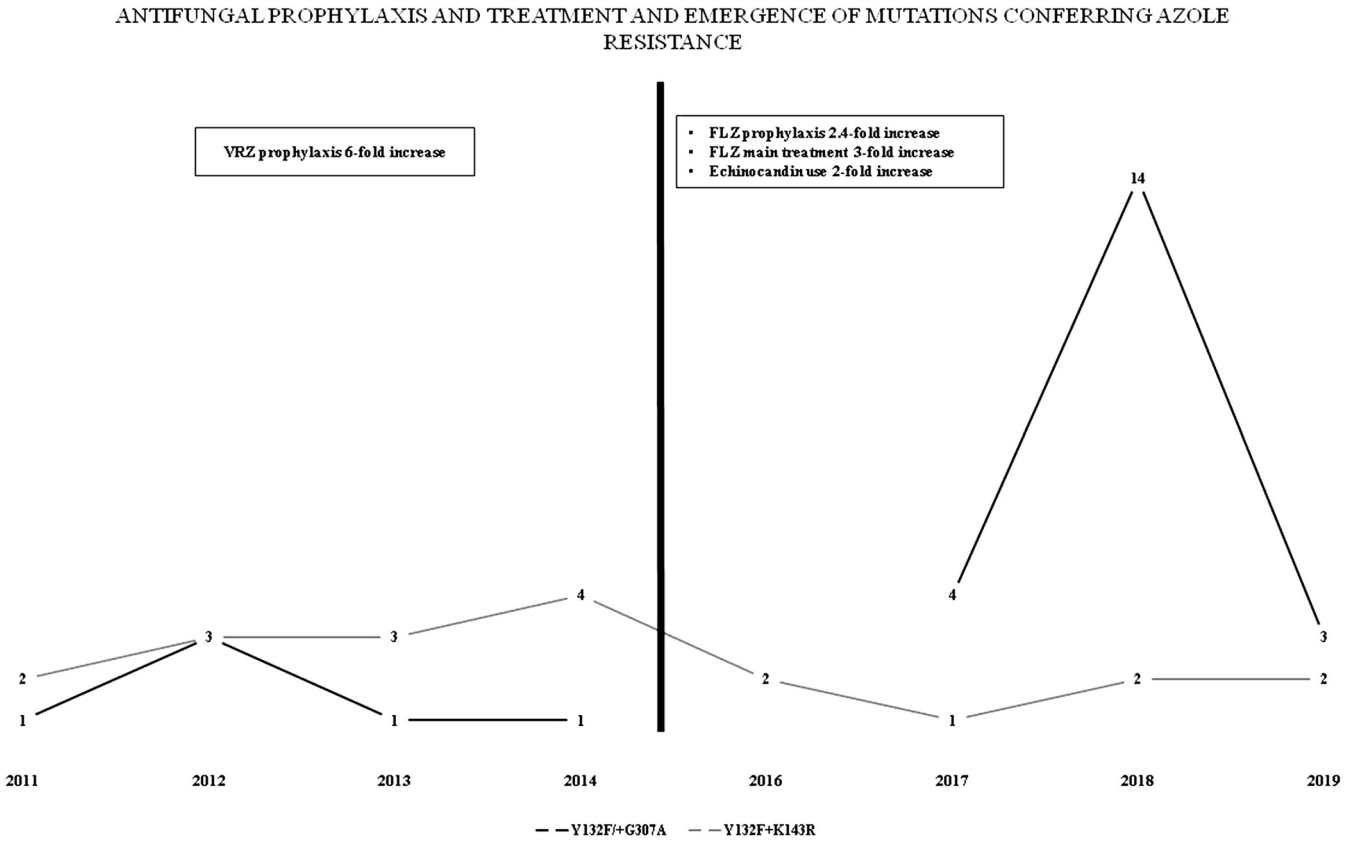

FIG 1 Frequency of mutations in *ERG11* responsible for resistance and azole use for prophylaxis or treatment in 2007 to 2019.

only two major genotypes, whereas several clusters were revealed by MST (Fig. S3 to S6). Therefore, all interpretations regarding genetic relatedness of the isolates were inferred from MST data. The first isolates carrying Y132F (108FS) and Y132F+K143R (106FS and 107FS) were detected in 2011 in different patients and showed a high degree of similarity (Fig. S3); among them, 106FS and 107FS were 100% identical (Table 1 and Fig. S3). Surprisingly, 63% of the FLZR isolates carrying either Y132F or Y132F+K143R ($n = 34$) were clustered into two main clades located close to one another (Table 1, Fig. 2, and Fig. S3). Approximately 95% of the isolates carrying Y132F+K143R ($n = 18$) and 16.6% of those harboring Y132F ($n = 4$) belonged to the same cluster and were recovered in 2011 to 2019. About 58% of the isolates carrying Y132F ($n = 14$) belonged to two distinct clusters ($n = 11$ [45.8%] and $n = 3$ [12.5%]) (Table 1, Fig. 2, and Fig. S3). As for their treatments, 48.8% of the patients infected with strains harboring Y132F alone and/or Y132F+K143R in Erg11 ($n = 21$) did not receive any azoles during their hospitalization periods. Among the FLZR isolates lacking *ERG11* mutations responsible for resistance, 50% ($n = 3$; 34R, 37R, and 38R) were clustered together and were recovered in 2013 from different wards, whereas the remaining isolates were scattered among different genotypic clusters. Azole-cross-resistant isolates were grouped in clusters A (84.2%; $n = 16$), C (10.5%; $n = 2$), and E (5.25%; $n = 1$) (Table 1 and Fig. S3). Clonality was also detected among FLZS isolates; the largest cluster contained 63 isolates, followed by other clusters containing 20, 19, and 13 isolates (Fig. S5).

Biofilm formation. Although the isolates significantly differed in biofilm-forming capacity, there was linear correlation between the results obtained with crystal violet and resazurin staining ($r^2 = 0.66$, $P < 0.001$). Isolates carrying the Y132F mutation in Erg11p produced significantly less biofilm than the other FLZR isolates ($P = 0.02$) (Fig. S7).

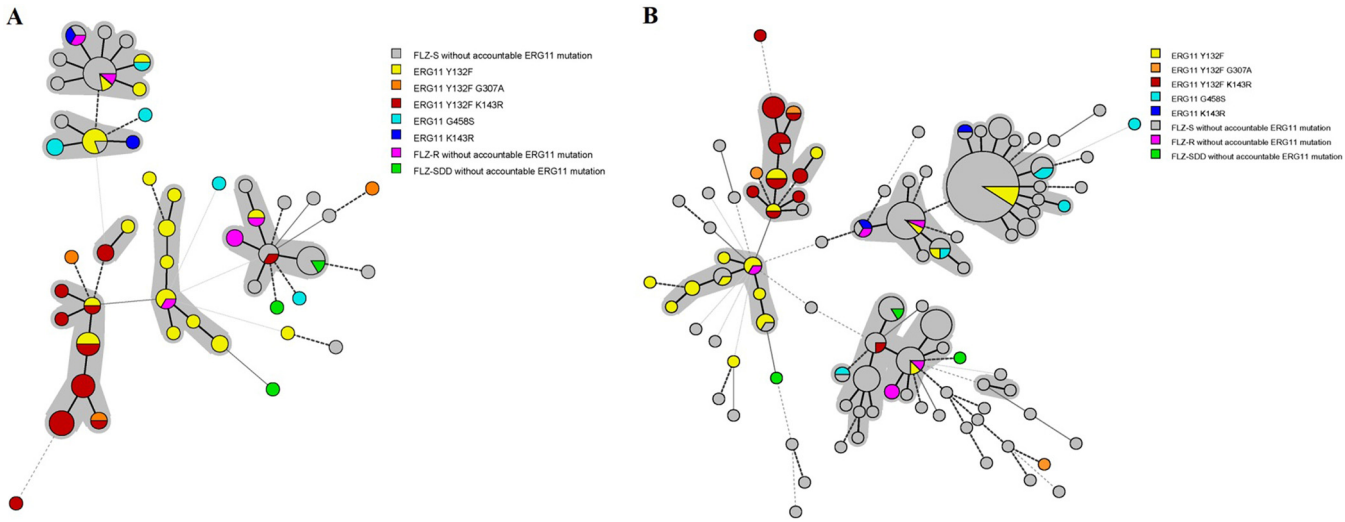

FIG 2 Minimum spanning tree obtained by MST of 91 isolates with azole resistance (A) and all study isolates (B). Isolates carrying Y132F and Y132F+K143R formed distinct clusters. SDD, susceptible (dose dependent).

DISCUSSION

A recent global study indicated that *C. parapsilosis* has the highest rate of FLZ resistance among *Candida* species, which has become a matter of growing concern, including in Europe (11). FLZ resistance rates significantly differ among countries and individual health care centers (11), emphasizing the need for active surveillance to prevent further expansion of FLZR *C. parapsilosis* in clinical settings. Interestingly, FLZR *C. parapsilosis* isolates are prevalent in three countries with the highest rate of FLZR *C. auris* (18), i.e., South Africa (5), India (6), and South Korea (7), further limiting the application of FLZ as first-line therapy. In this study, we report a clonal outbreak of FLZR *C. parapsilosis* in 2007 to 2019 in Ege University Hospital (Izmir, Turkey), which was especially characteristic of pediatric wards.

Overall, 26.5% and 8.3% of isolates were FLZR and VRZR, respectively; all of the latter were also cross-resistant to FLZ. Most FLZR isolates (90%) carried previously reported Erg11p mutations, and 5% of them carried a new one (G307A). Among the reported mutations, Y132 F and K143R have been detected in FLZR *C. parapsilosis* (6) and G458SS in FLZR *C. orthopsilosis*, a sibling species to *C. parapsilosis* (17). Although previous studies indicate that the Mrr1p mutations G583R and K873N (19) and L986P (20) are associated with FLZ and/or VRZ resistance, none of our isolates harbored them. Similar to *Candida albicans* (21), residues located near the C terminus of Mrr1 (926 and 1027) and Tac1 (760, 761, 803, 956, and 966) might contribute to azole resistance (21). Azole resistance mechanisms in *C. parapsilosis*, unlike those in *C. albicans*, are not well characterized, and we hope that the repertoire of mutations found in our study inspire heterologous expression studies in the future to broaden our knowledge on this growing problem.

The observed overall mortality rate in our study (38.1%) is similar to those reported in Brazil (22, 23), the United States (24), Portugal (25), and Italy (26) (30 to 46%) and 2.7 times higher than that reported in Taiwan (14%) (27). Importantly, we observed a link between mutations in genes implicated in azole resistance and mortality. Surprisingly, the mortality rate due to isolates with Y132F was 3 times higher than that caused by isolates with Y132F+K143R (OR = 6.8; $P = 0.005$). Experiments involving *Galleria mellonella* larvae infected with wild-type (WT) and *C. parapsilosis* isolates with Y132F showed a higher virulence when fluconazole was used for treatment (28). However, the impact of various *ERG11* mutations on virulence of mutated and WT isolates has not been tested when *Galleria* infected with respective isolates is not treated with azoles. Although it has been shown that GOF mutation in Upc2 decreases the virulence of *C.*

albicans (29), considering the increasing number of reported isolates with Y132F and considering that association of Tac1, Mrr1, and Upc2 with virulence is relatively unknown in *C. parapsilosis*, our findings may deserve detailed investigation *in vivo*.

C. parapsilosis can acquire azole resistance either by selective pressure due to azole use or by horizontal acquisition of azole-resistant *C. parapsilosis* isolates in antifungal-naive patients (7). Therefore, we analyzed patients' treatment regimens and clonality of the isolates. Interestingly, the increasing FLZ use from 2015 onward paralleled an increasing frequency of isolation of *C. parapsilosis* isolates carrying Y132F. This finding is consistent with a study in Brazil showing that patients with clonal FLZR *C. parapsilosis* isolates carrying Y132F in Erg11p were previously exposed to FLZ (30). Moreover, significant positive correlation between nonsusceptibility of *Candida* species to azoles and FLZ use has been documented previously (31). Therefore, we speculate that the selective pressure exerted by azole use has partly resulted in emergence of FLZR *C. parapsilosis* in our hospital. To test the second idea, we assessed the genetic relatedness of FLZR isolates in relation to their treatment with azoles. Assessment of FLZR isolates by lineage using AFLP revealed two major clades with a high degree of similarity at the genome level. MST showed higher resolution and separated isolates into seven clusters, demonstrating that FLZR and FLZS isolates grouped in distinct clusters and accumulated over time. These observations suggest that AFLP does not have sufficient resolution to separate *C. parapsilosis* isolates and that MST should be the preferred method for clonal analysis of strains responsible for infection outbreaks. The overall high Simpson index value of the MST assay used here was demonstrated previously (32), and the fact that MST is being increasingly used for the genotyping assessment of clinically important fungi (33, 34), including *C. parapsilosis* (6), further suggests the reliability of this technique when dealing with outbreak scenarios. Moreover, the conspicuous grouping of FLZR and FLZS isolates into separate clusters and the finding that almost 50% of patients infected with FLZR isolates with Y132F/Y132F+K143R never received azoles may suggest an ongoing clonal outbreak of *C. parapsilosis* in our hospital that requires strict infection control and active environmental screening to identify and eradicate the source of infection.

It has been speculated that FLZR *C. parapsilosis* isolates with Y132F tend to be more clonal compared to other FLZR isolates and are more persistent in clinical settings (7). MST analysis found that both FLZR and FLZS isolates formed clonal clusters containing isolates recovered from 2007 to 2019. Moreover, biofilm formation, an index of persistence in hospital settings, was lower for isolates harboring Y132F than those without it, which is consistent with a study in Brazil showing that FLZR *C. parapsilosis* isolates with Y132F produced less biofilm than FLZS *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* (30). Collectively, these observations argue against the notion that isolates with Y132F are more persistent than the other FLZR or FLZS *C. parapsilosis* isolates. Although it is tempting to attribute this phenomenon to a fitness cost posed by Y132F, isolates with the double mutation Y132F+K143R had a higher biofilm production. Therefore, dedicated studies are required to verify this finding *in vivo* and to identify the subcellular mechanisms involved.

Candida parapsilosis has been reported to respond to high concentrations of echinocandins when tested *in vitro*, which is attributed to a natural polymorphism in the *FKS1* gene (35). However, in agreement with clinical studies conducted in the United States (36) and Spain (37), we did not observe a significant difference in the outcome for patients treated with FLZ or echinocandins. Of note, the comparative efficacy of azoles and echinocandins was not the focus of this study; such a study should take into account the severity of the disease and the underlying conditions. The alarming increase in the number of fatalities due to azole-resistant *C. parapsilosis* carrying the Y132F mutation, which could be further aggravated by clonal expansion in our hospital, together with the overall low resistance of *C. parapsilosis* to echinocandins (3, 11) reinforces the suitability of echinocandins for treatment of *C. parapsilosis* bloodstream infections. However, one should consider the possibility that the high *in*

vitro MICs of anidulafungin for *C. parapsilosis* may hamper its efficacy in the clinical setting.

In conclusion, the observed clonal outbreak of fatal infections due to FLZR *C. parapsilosis* in our hospital, especially in pediatric wards, is worrisome and may be the consequence of inappropriate application of antifungal drugs and the lack of strict infection control measures, including hand hygiene. Therefore, active environmental surveillance followed by establishing strict infection control strategies and rigid sanitation standards are necessary to confine the spread of this pathogen. Moreover, implementation of appropriate antifungal therapy limiting the emergence of FLZR isolates is of paramount importance.

The retrospective nature of our study was one of its main limitations; as a result, we could not obtain clinical data for some patients. Also, the contribution of GOF mutations in *TAC1*, *MRR1*, and *UPC2* to azole resistance, which has mainly been studied in *C. albicans*, is not fully recapitulated in *C. parapsilosis* (10); therefore, future studies in this direction are warranted.

MATERIALS AND METHODS

Study design, definitions, and identification. The study included all patients with candidemia due to *C. parapsilosis* admitted to Ege University Hospital, Izmir, Turkey, from 2007 to 2019. Being among the five largest hospitals in Turkey with 1,816 beds, Ege University Hospital admits 67,000 inpatients and 1,200,000 outpatients annually. Although species identity was considered, neutropenic patients with fever and sepsis were treated with caspofungin or amphotericin B (AMB). Positive blood cultures (100 μl) were streaked on Sabouraud dextrose agar and incubated at 37°C for 24 to 48 h, and the single colonies obtained were stored at −80°C. All isolates were primarily identified using the API 20C AUX system (bioMérieux, Marcy l'Etoile, France) and further characterized using the MALDI Biotyper system (Bruker Daltonik, Bremen, Germany) with a full extraction method (38). This study was approved by the ethics committee of Ege University Hospital (approval number 20-2T/30).

Antifungal susceptibility testing. Susceptibility to FLZ (Sigma-Aldrich, St. Louis, MO, USA) and VRZ (Sigma) was tested by the broth microdilution method according to CLSI document M27-A3 (39). Plates were incubated at 35°C for 24 h and MICs were determined by visual examination. MICs of ≥8 mg/liter and ≥1 mg/liter were considered to indicate resistance to FLZ and VRZ, respectively (40), while isolates for which FLZ and VRZ MICs were 4 mg/liter and 0.25 to 0.5 mg/liter were considered susceptible (dose dependent) and intermediate, respectively (40). Isolates with FLZ and VRZ MICs of ≤2 mg/liter and ≤0.12 mg/liter, respectively, were considered susceptible (40). *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used for quality control.

DNA extraction, sequencing, and genotyping. DNA extraction followed a cetyltrimethylammonium bromide (CTAB) protocol (38). Primers for *ERG11*, *TAC1*, *UPC2*, and *MRR1* genes are shown in Table S1. Sequences were assembled using SeqMan Pro software (DNASTAR, Madison, WI, USA) and after curation were aligned to WT (41) *ERG11* (GQ302972), *MRR1* (HE605205), *TAC1* (HE605204), and *UPC2* (HE605206).

The genotypic diversity and genetic relatedness of the isolates were assessed by AFLP (42) and MST (32) as previously described.

Biofilm formation and quantification. *In vitro* biofilm formation was assessed for 11 strains harboring Y132F and 9 strains lacking this mutation but resistant to FLZ (Table S2) (7, 43, 44). Biofilms were formed in 96-well microtiter plates for 24 h and stained with crystal violet or resazurin (CellTiter-Blue; Promega, Madison, WI, USA) as previously described (45). Absorbance (crystal violet) and fluorescence (resazurin) were measured using an Envision microtiter plate reader (Perkin Elmer, Waltham, MA, USA).

Statistical analysis. Clinical and microbiological data were evaluated using SPSS v24 (SPSS Inc., Chicago, IL, USA). Biofilm formation was compared using an independent-samples *t* test. The association between two nominal variables of mutations and survival was assessed using Phi and Cramer's V.

Data availability. The sequences determined in this study for *ERG11* (MK924157 to MK924381), *MRR1* (MT429530 to MT429618), *TAC1* (MK940393 to MK940481), and *UPC2* (MT429619 to MT429707) were deposited in GenBank.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 9.5 MB.

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