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Genetic relatedness among azole-resistant *Candida tropicalis* clinical strains in Taiwan from 2014 to 2018

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ABSTRACT

To monitor trends in the distribution of yeast species and the susceptibilities of these species to commonly prescribed antifungal drugs, we conduct the Taiwan Surveillance of Antimicrobial Resistance of Yeasts (TSARY) every 4 years. We found that 25 of 294 *Candida tropicalis* isolates from TSARY 2014 and 31 of 314 *C. tropicalis* isolates from TSARY 2018 were resistant to fluconazole. We determined the genetic relatedness among fluconazole-resistant *C. tropicalis* isolates by multilocus sequence typing (MLST). Among 174 *C. tropicalis* isolates, including all 56 fluconazole-resistant, all 26 susceptible-dose dependent and 92 selected fluconazole-susceptible isolates, 59 diploid sequence types (DSTs) were identified. We found that 22 of the 25 fluconazole-resistant *C. tropicalis* from TSARY 2014 and 29 of the 31 fluconazole-resistant *C. tropicalis* from TSARY 2018 were genetically related and belonged to the same cluster (clade 4). A combination of mutation and overexpression of *ERG11*, encoding the target of azole drugs, was the major mechanism contributing to drug resistance. Approximately two-thirds of reviewed patients infected or colonised by fluconazole-resistant *C. tropicalis* were azole-naïve. Furthermore, there was no evidence of patient-to-patient transmission. Because the clade 4 fluconazole-resistant *C. tropicalis* strain persists in Taiwan, it is important to identify the source of azole-resistant *C. tropicalis* to prevent the spread of this resistant strain.

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1. Introduction

Fungal infections pose a serious threat to patients with conditions that cause immunosuppression [1–4]. The prevalence of fungal infections has increased significantly in recent decades because of the expanding number of immunocompromised patients, increased use of invasive medical devices and extensive use of broad-spectrum antibiotics [5–7]. Although the problem is worldwide, the possibility of regional differences in such infections points to the importance of areas conducting their own epidemiological studies of fungal infections, providing updated local data for appropriate clinical therapies [8–10].

Candida species are a leading aetiological cause both of local and systemic fungal infections associated with significant morbidity and mortality. Among the *Candida* species that can cause candidiasis in humans, the five most common are *Candida albicans, Candida glabrata, Candida tropicalis, Candida parapsilosis* and *Candida krusei* [6,11,12].

To monitor the trends in species distribution and drug susceptibility of clinical yeast pathogens, in 1999 we initiated a national survey, the Taiwan Surveillance of Antimicrobial Resistance of Yeasts (TSARY). To date, six TSARYs have been conducted. The species distribution and drug susceptibilities of *Candida* species isolates collected in 1999, 2002, 2006, 2010 and 2014 have been released in respective reports [13–17]. We found that *C. tropicalis* is the most common non-*albicans Candida* species causing diseases in Taiwan [12]. Azoles are among the most commonly prescribed drugs for treating systemic fungal infections. The emergence of azole-resistant fungal pathogens is a growing concern for the medical community [18,19]. We reported that 5 (0.5%) and 31 (2.8%) isolates characterised in TSARY 2010 and 2014, respectively, were resistant to fluconazole [17]. There was a significant increase in the azole resistance rate between 2010 and 2014. This was especially the case for *C. tropicalis*, with none of its 270 samples displaying resistance to fluconazole in 2010, but 25 of its 294 samples doing so in 2014 [17].

In the present study, we first determined the azole susceptibilities of *C. tropicalis* from TSARY in 2018 and then investigated the genetic relatedness among fluconazole-resistant *C. tropicalis* from 2014 and 2018. We also investigated molecular mechanisms contributing to drug resistance and possible routes for patients acquiring fluconazole-resistant *C. tropicalis*.

2. Materials and methods

2.1. Isolates

Candida tropicalis isolates were collected as part of TSARY. The 2018 collection protocol and identification of *C. tropicalis* were the same as in 2014 [17]. In brief, each hospital was asked to submit all yeast pathogens from sterile sites as well as up to 10 *C. albicans* and 40 non-*C. albicans* yeast isolates with clinical significance from non-sterile sites. In principle, only one isolate was accepted from each specimen. Nevertheless, when there were multiple species isolated from one specimen or multiple specimens obtained from same patient, only one isolate of the same species obtained from the same body site of an individual patient was analysed.

2.2. Antifungal susceptibility testing

The drug susceptibilities of *C. tropicalis* isolates were determined by the in vitro antifungal susceptibility testing procedure established in our laboratory [17], which follows the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [20]. Growth of each isolate was measured using a Multiskan^{TM} FC Microplate Photometer (Thermo Fisher Scientific Inc., USA) following incubation at 35°C for 24 h.

The final concentrations of ranged from 0.125 mg/L to 64 mg/L for fluconazole and from 0.0156 mg/L to 8 mg/L for voriconazole. Minimum inhibitory concentrations (MICs) were defined as the concentration of drug capable of reducing the turbidity of cells by more than 50%. The CLSI newly defined species-specific breakpoints [21] were applied. For fluconazole, the clinical MIC breakpoints were as follows: ≤2 mg/L, susceptible; 4 mg/L, susceptible-dose dependent (SDD); and ≥8 mg/L, resistant. For voriconazole, the clinical MIC breakpoints were: ≤0.12 mg/L, susceptible; 0.25–0.5 mg/L, intermediate; and ≥1 mg/L, resistant.

2.3. Multilocus sequence typing (MLST)

MLST was conducted as described in our previous report [22]. Phylogenetic analysis was performed using the unweighted pair-group method with arithmetic average (UPGMA), which was created using MEGA X software. A cut-off *P* distance of 0.01 was chosen because it separated clades that contained known examples of isolates. We constructed a diploid sequence type (DST)-based phylogenetic tree of global *C. tropicalis* composed of 1116 DSTs according to the *C. tropicalis* MLST database, and clades containing more than 10 genetically closely related DSTs were labelled. The primers used are listed in Supplementary Table S1.

2.4. Qualitative analysis of the transcription level of genes involved in drug resistance by real-time PCR

We further determined the expression levels of *CDC1, ERG1* and *MDR1*. Cells were harvested at an optical density of 600 nm (OD_{600}) of 0.7–0.9 after growing in yeast extract–peptone–dextrose (YPD) liquid medium at 30°C for 6 h. Expression of *ACT1* in each strain was used to normalise expression of the three other genes. The mRNA level of a fluconazole-susceptible isolate YFA120877 was used as the denominator for normalisation. The primers used in the present study are listed in Supplementary Table S1.

3. Results

3.1. Chart review of fluconazole-resistant Candida ***tropicalis*** from TSARY in 2014

Approximately 8.5% of *C. tropicalis* collected by TSARY in 2014 were resistant to fluconazole. To investigate whether fluconazole-resistant *C. tropicalis* isolates were selected within patients under clinical antifungal drug selection, we reviewed the clinical characteristics of the 25 patients infected or colonised by fluconazole-resistant strains (Table 1). As expected, most of the patients had multiple underlying co-morbidities. There was neither clustering nor a statistically significant relationship of the cases to time or place. Hence, there was no evidence of patient-to-patient transmission. Interestingly, most (18/25; 72%) had not received azole antifungal agents within 6 months prior to *C. tropicalis* isolation. Furthermore, 3 of the 11 deaths were candidiasis-related.

3.2. Genetic relatedness among fluconazole-resistant Candida tropicalis from TSARY in 2014

Using MLST, we investigated whether the 25 fluconazole-resistant *C. tropicalis* collected by TSARY in 2014 were genetically related. This revealed the genome types of all 25 fluconazole-resistant, all 22 SDD and 31 hospital and/or body site type matched susceptible *C. tropicalis* isolates. There were 7 and 15 fluconazole-resistant isolates belonging to DST225 and DST506, respectively

Table 1
Characteristics of 25 patients infected or colonised by fluconazole-resistant *Candida tropicalis* isolates.

Patient	Strain	Sex/age (years)	District [a]; type of hospital and ward [b,c]	Co-morbidities	Sample	*Candida* disease	Prior azole exposure within 6 months	Sequential antifungal treatment (days)	Outcome at discharge or 4 weeks	Candidiasis-associated mortality	DST	Erg11-132[d]	Erg11-154[d]	*ERG11* [e]
1	YM141153	M/57	S; C, ICU	MDS w/ AML	Blood	BSI	Yes	VRC (12)	Died	Yes	225	YF	SF	4.02
2	YM140285	M/80	C; C, INX	Lung Ca, PN, COPD, CVA, DM, HTN, PAOD	Urine	UTI	Yes	FLU (15)	Died	No	506	YF	SF	2.58
3	YM140292	M/61	C; C, INX	CAD	Pus	SSTI	No	FLU (21)	Alive	No	506	YF	SF	3.03
4	YM140298	M/66	C; C, INX	PN, CKD, COPD	Urine	UTI	Yes	FLU (7)	Died	No	585	YY	SS	0.67
5	YM140316	M/84	C; C, ICU	Open fracture of lower limb, CAD, DM	Pus	Colonisation	No	No	Died	No	506	YF	SS	4.07
6	YM140896	M/51	C; R, INX	NPC w/ liver mets, HTN	Blood	BSI	Yes	AND (7)	Died	No	911	YY	SS	1.06
7	YM140132	F/78	S; R, ICU	Lung Ca, UTI (*Proteus* sp.)	Urine	Colonisation	No	No	Alive	No	506	YF	SF	3.08
8	YM140136	M/40	S; R, ICU	HIV, PN	Urine	UTI	Yes	No	Died	No	225	YF	SF	4.42
9	YM140566	F/80	S; R, INX	VHD, DM, HTN	Urine	UTI	No	FLU (8)	Died	No	506	YF	SF	4.07
10	YM140586	M/72	E; R, ICU	Rectal Ca, DM	Blood	BSI	No	No	Alive	No	225	YF	SF	4.56
11	YM140258	M/54	S; C, ICU	CML, PN, neutropenia	Blood	BSI	Yes	FLU (2), AmB (1), MCF (3)	Died	Yes	506	YF	SF	1.61
12	YM140840	M/50	E; R, INX	LC w/ EV bleeding	Urine	Colonisation	No	No	Alive	No	506	YF	SF	3.2
13	YM140663	M/62	C; C, ICU	PN, DM, HTN	Urine	Colonisation	No	No	Alive	No	225	FF	FF	3.7
14	YM140939	M/79	C; R, ICU	Pancreatic Ca, DM, HTN	CVP tip	Susp. CR-BSI	No	FLU (16)	Died	Yes	506	YF	SF	3.15
15	YM140945	F/75	C; R, OUT	HTN	Urine	Colonisation	No	No	Alive	No	225	YF	SF	3.31
16	YM140372	M/76	N; R, INX	PN, CAD, COPD, DM	Sputum	Colonisation	No	No	Alive	No	225	YF	SF	4.59
17	YM141048	M/82	S; C, INX	PN	Urine	Colonisation	No	No	Alive	No	225	YF	FF	5.31
18	YM141055	F/92	S; C, INX	PN, HTN	Sputum	Colonisation	No	No	Alive	No	153	YY	SS	1.24
19	YM140441	F/79	C; R, ICU	UTI (*Klebsiella* sp.), DM, HTN	Urine	Colonisation	No	No	Alive	No	506	YF	SF	4.88
20	YM140442	F/40	C; R, OUT	None	Urine	Colonisation	No	No	Alive	No	506	YF	SF	3.84
21	YM140448	M/83	C; R, ICU	COPD	Sputum	Colonisation	No	FLU (12)	Alive	No	506	FF	SF	4.68
22	YM140455	M/72	C; R, ICU	Bacteraemia, CAD, HTN	Sputum	Colonisation	No	No	Died	No	506	YF	SF	3.11
23	YM140055	M/50	E; C, INX	NF, HCV, LC w/ EV	CVP tip	Susp. CR-BSI	Yes	FLU (9)	Alive	No	506	YF	SF	3.76
24	YM140038	M/59	E; C, OUT	HTN	Wound	Colonisation	No	No	Alive	No	506	YF	SF	4.76
25	YM140066	M/82	E; C, ICU	PN, ESRD on PD, CVA, DM, HTN	Ascites	Peritonitis	No	FLU (6)	Died	Yes	506	YF	SF	5.44

AmB, amphotericin B; AND, anidulafungin; BSI, bloodstream infection; Ca, cancer; CAD, coronary artery disease; CKD, chronic kidney disease; CML, chronic myeloid leukaemia; COPD, chronic obstructive pulmonary disease; CR-BSI, catheter-related bloodstream infection; CVA, cerebrovascular accident; CVP, central venous catheter; DM, diabetes mellitus; DST, diploid sequence type; ESRD on PD, end-stage renal disease on peritoneal dialysis; FLU, fluconazole; HCV, hepatitis C virus infection; HIV, human immunodeficiency virus infection; HTN, hypertension; LC w/ EV, liver cirrhosis with oesophageal varices; MCF, micafungin; MDS w/ AML, myelodysplastic syndrome with acute myeloid leukaemia; NF, necrotizing fasciitis; NPC w/ liver mets, nasopharyngeal cancer with liver metastasis; PAOD, peripheral arterial occlusion disease; PN, pneumonia; SSTI, skin and soft-tissue infection; Susp., suspected; UTI, urinary tract infection; VHD, valvular heart disease; VRC, voriconazole.

[a] Districts N, C, S and E indicate northern, central, southern and eastern Taiwan, respectively.

[b] Hospital types C and R indicate medical centres and regional hospitals, respectively.

[c] Wards ICU, INX and OUT indicate intensive care units, non-ICU inpatients and outpatients, respectively.

[d] Amino acid at the 132 and 154 positions of the Erg11 protein; F, phenylalanine; S, serine; Y, tyrosine.

[e] Related level of mRNA.

(Table 2). According to the phylogenetic tree composed of 1116 DSTs, DST225 and DST506 are closely related, with one nucleotide different on *XYR1* and both belonging to clade 4. Of the 22 clade 4 fluconazole-resistant isolates, 1, 5, 6 and 10 isolates were from hospitals throughout Taiwan (1 northern, 3 eastern, 5 southern and 4 central hospitals, respectively). Hence, these fluconazole-resistant *C. tropicalis* were not from an outbreak from a single hospital or region.

3.3. Mechanisms contributing to fluconazole resistance

The Erg11 of all three non-clade 4 fluconazole-resistant isolates was wild-type, whereas all 22 clade 4 fluconazole-resistant isolates contained Y132F and/or S154F mutations of Erg11. We found that all clade 4 fluconazole-resistant isolates, except YM140258, had a two-fold or greater mRNA level of *ERG11* than a fluconazole-susceptible control strain. On the other hand, the mRNA levels of *CDR1* and *MDR1* were not increased. This suggests that overexpression of the fluconazole mutated target, Erg11, is the molecular mechanism contributing to fluconazole resistance of the clade 4 strain.

3.4. Azole susceptibilities of Candida tropicalis from TSARY in 2018

To determine whether the clade 4 fluconazole-resistant *C. tropicalis* strain persists in Taiwan, we conducted TSARY again in 2018. Of 314 *C. tropicalis* tested isolates, 31 (9.9%) *C. tropicalis* isolates (including 15 from urine, 5 from blood, 4 from sputum, 2 each from

Table 2
Distribution of genotypes of 174 *Candida tropicalis* isolates.

	TSARY 2014				TSARY 2018				
Clade/DST	S	SDD	R	Subtotal	S	SDD	R	Subtotal	Total
Clade 4									
225	0	13	7	20	0	3	3	6	26
506	0	0	15	15	0	0	21	21	36
508	0	0	0	0	0	1	0	1	1
546	0	0	0	0	0	0	2	2	2
592	0	0	0	0	0	0	1	1	1
595	0	1	0	1	0	0	0	0	1
600	0	1	0	1	0	0	0	0	1
667	1	0	0	1	0	0	0	0	1
879	0	0	0	0	0	0	1	1	1
921	1	0	0	1	0	0	0	0	1
924	0	1	0	1	0	0	0	0	1
1096	0	0	0	0	0	0	1	1	1
Subtotal	2	16	22	40	0	4	29	33	73
Clade 5									
98	0	0	0	0	2	0	0	2	2
140	12	0	0	12	18	0	0	18	30
181	0	0	0	0	1	0	0	1	1
357	1	0	0	1	0	0	0	0	1
443	0	0	0	0	1	0	0	1	1
797	0	0	0	0	1	0	0	1	1
910	2	0	0	2	0	0	0	0	2
911	1	0	1	2	1	0	0	1	3
922	0	1	0	1	0	0	0	0	1
953	0	0	0	0	2	0	0	2	2
954	0	0	0	0	2	0	0	2	2
955	0	0	0	0	1	0	0	1	1
958	0	0	0	0	1	0	0	1	1
Subtotal	16	1	1	18	30	0	0	30	48
Other 33 DSTs	13	5	2 (153 & 585) [a]	20	31	0	2 (1095 & 1141)	33	53
Total	31	22	25	78	61	4	31	96	174

DST, diploid sequence type; R, resistant; S, susceptible; SDD, susceptible-dose dependent; TSARY, Taiwan Surveillance of Antimicrobial Resistance of Yeasts.
[a] Number of isolates (DSTs).

ascites and wound, and 1 each from catheter tip, ear and pus) were resistant to fluconazole, among which 29 were cross-resistant to voriconazole. Approximately 8.9% (5/56) of isolates from blood and 10.1% (26/258) of isolates from non-blood sites were fluconazole-resistant. Thus, the fluconazole resistance rate of *C. tropicalis* from blood was similar to that from non-blood sites. Among the 31 fluconazole-resistant *C. tropicalis*, 2 were found in one eastern Taiwan hospital, while 5 were from three southern hospitals, 12 from four northern hospitals and 12 from five central hospitals. As in the case of the isolates collected in TSARY in 2014, those fluconazole-resistant *C. tropicalis* were distributed throughout Taiwan.

3.5. Molecular typing of Candida tropicalis from TSARY in 2018

Fluconazole-resistant *C. tropicalis* isolates were detected in TSARY in 1999, 2006, 2014 and 2018, but not in 2002 or 2010. Thus, the most recent survey marks the first time that fluconazole-resistant *C. tropicalis* was detected in two successive TSARYs. We further investigated whether the clade 4 fluconazole-resistant strain has persisted in Taiwan since 2014. We determined the genome types of all 31 fluconazole-resistant, all 4 SDD and 61 hospital and/or body site type matched susceptible *C. tropicalis*. We found that 29 fluconazole-resistant isolates (21 DST506, 3 DST225, 2 DST546, and 1 each of DST592, DST879 and DST1096) belonged to the same clade 4 as well as 1 each of DST1095 and DST1141.

In the present study, of the 174 tested isolates from TSARY both in 2014 and 2018, 59 DSTs were identified. It is worth noting that the 91.1% of fluconazole-resistant *C. tropicalis* were clustered together and belonged to clade 4, whereas fluconazole-susceptible isolates were diverse and belonged to 46 other DSTs (Table 2; Supplementary Fig. S1). Among the 174 tested isolates, two large clusters were identified: clade 4, which contained most of the fluconazole-resistant isolates; and clade 5, which contained 48 isolates.

4. Discussion

Continuous exposure to azoles may have a major impact on selecting fluconazole-resistant *Candida* species. Our findings in Taiwan point to the importance of *C. tropicalis* cross-resistance to azole drugs. Importantly, this phenomenon of azole resistance in *C. tropicalis* has been observed not only in Taiwan but also elsewhere [4,23,24].

In Taiwan, fluconazole-resistant clade 4 *C. tropicalis* isolates have also been detected in candidaemia patients in a non-TSARY hospital [25]. Furthermore, a report from China found that the majority (23/30) of fluconazole-resistant *C. tropicalis* samples also belonged to clade 4 [26]. A combination of two mechanisms involved in azole resistance, mutation and overexpression of the azole target, may help this clade 4 strain persist in patients and environments in the presence of azole drugs. Although the data of our clinical chart review do not support that patient-to-patient transmission of fluconazole-resistant isolates occurs, we still cannot rule out the possibility of inter-human horizontal transmission. Therefore, it is important to investigate the sources of the clade 4 strain and to understand the dissemination of this strain in Taiwan and other countries.

There are at least two major routes for acquiring azole-resistant *C. tropicalis*. One is selection pressure within patients from clinical azole drug use. The other is that a patient acquires azole-resistant *C. tropicalis* from the environment. In the present study, we found

no evidence of patient-to-patient transmission. However, we cannot rule out the possibility that another unknown human source is involved, such as has been reported for *C. parapsilosis* [27–30]. Although not generally airborne, like multidrug-resistant *Candida auris* causing outbreaks in healthcare settings [31], azole-resistant *C. tropicalis* may colonise humans through direct contact or through food and water in the community. In the present study, approximately two-thirds of patients infected or colonised by fluconazole-resistant *C. tropicalis* had not received azole antifungal agents prior to *C. tropicalis* isolation. Furthermore, fluconazole non-susceptible *C. tropicalis* has been detected on the surface of fruits [32]. Preliminary data from the Surveillance of Pathogenic Yeasts in Taiwan Hospital Environments show that the clade 4 fluconazole-resistant *C. tropicalis* existed in hospital environments. These observations, together with reports that azole-resistant *Aspergillus fumigatus* recovered from soil and compost were genetically related to clinical resistant isolates [33], raised the possibility that these azole-resistant *C. tropicalis* might account for resistant strains in humans. Because clade 4 fluconazole-resistant *C. tropicalis* has been detected in hospital environments, clinical interventions such as handwashing and environmental sterilising can be implemented to help eliminate the risk of patients acquiring it. Moreover, to investigate the transmission routes of azole-resistant *C. tropicalis* between patients and environments, a survey of different environments, such as animals or food from supermarkets and/or farms, is required to determine the presence of clade 4 azole-resistant *C. tropicalis*. Based on the concept of 'One Health' [34], whether the clade 4 azole-resistant *C. tropicalis* was originally selected within patients by clinical azole drugs or originated from environments with agricultural fungicides requires further investigation.

Although MLST is an important and convenient tool for studying the genetic relatedness/diversity of isolates as well as allowing comparison of global data, the evaluation of only DNA fragments of a few genes has potentially serious limitations compared with study of the whole genome. Other methods, such as mitochondrial DNA sequencing and/or whole-genome sequencing, should be applied to further assess whether isolates belonging to clade 4 are clonal. It would also be interesting to investigate the evolution of different genotypes of *C. tropicalis*. Although the limitations of MLST prevented discovery of whether those isolates of clade 4 are clonal, our findings still warrant warning medical mycologists that a potential *C. tropicalis* strain persists in Taiwan and may be causing infections in other areas.

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Competing interests

None declared.

Ethical approval

The TSARY project was approved by the Research Ethics Committee at the National Health Research Institutes [EC1030509-E].

Sequence information

Not applicable.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2022.106592.

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