



Antifungal Susceptibility of Clinical Yeast Isolates from a Large Canadian Reference Laboratory and Application of Whole-Genome Sequence Analysis To Elucidate Mechanisms of Acquired Resistance

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ABSTRACT To understand the epidemiology and susceptibility patterns of yeast infections in Ontario, Canada, we examined 4,715 clinical yeast isolates submitted to our laboratory for antifungal susceptibility testing from 2014 to 2018. *Candida albicans* was the most frequently submitted species (43.0%), followed by *C. glabrata* (21.1%), *C. parapsilosis* (15.0%), and *C. tropicalis* (6.2%). Twenty-three other *Candida* spp. (11.6%) and 4 non-*Candida* species (3.1%) were also identified. Few changes in species distribution were observed from 2014 to 2018, but the total numbers of yeast isolates sent for testing increased, with an annual 7.4% change. According to CLSI clinical breakpoints, resistance rates remained low overall. Moderate fluconazole resistance was noted among *C. glabrata* (9%), *C. parapsilosis* (9%), and *C. tropicalis* (12%) isolates. Only 1% of *C. glabrata* isolates were resistant to caspofungin, micafungin, and anidulafungin. Whole-genome sequence analysis confirmed 11 cases of acquired resistance to azoles or echinocandins via in-host evolution. There were mutations in the gene for the catalytic subunit of 1,3-beta-glucan synthase-mediated echinocandin resistance in 3 of 3 *C. albicans* strains, 3 of 4 *C. glabrata* strains, and 1 strain of *C. tropicalis*. Azole resistance was likely caused by a homozygous *ERG3* mutation in 1 *C. albicans* strain and a previously undescribed chromosomal-duplication event involving *ERG11* and *TAC1* orthologs in 1 *C. tropicalis* strain. While antifungal resistance rates remain low among yeast isolates in Ontario, ongoing surveillance is necessary to inform empirical therapy for optimal patient management and to guide antifungal stewardship.

KEYWORDS antifungal resistance, antifungal susceptibility testing, azole, *Candida*, echinocandin, whole-genome sequencing, yeast

Invasive fungal infections are a significant cause of morbidity and mortality (1, 2). Among the fungi that can cause invasive fungal infections, *Candida* yeast species are the primary threat, particularly in health care settings, where they are among the top four most common nosocomial bloodstream pathogens (1, 3–5). Other yeasts, such as *Saccharomyces* spp., *Trichosporon* spp., *Malassezia* spp., *Geotrichum candidum*, and *Rhodotorula* spp., have also been implicated in invasive fungal infections but remain relatively rare (2, 6).

Several studies have described a changing epidemiology of candidiasis. Although species distributions of clinical yeast isolates differ based on geography and population, there has been a notable shift away from *Candida albicans* to non-*albicans* species; species such as *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* are increasingly being

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encountered. Increased utilization of antifungal drugs for both treatment and prophylaxis in recent years has been identified as a cause of the changing epidemiology of candidiasis through selective pressure favoring resistant species (1, 7, 8). Overall, antifungal resistance remains relatively low; however, instances of resistance are increasingly being reported worldwide (9, 10). There has been a perceptible increase in resistance, particularly to azoles, among *C. glabrata* isolates in North America and *C. tropicalis* isolates in Asia (1, 11–14). *C. glabrata* isolates are generally susceptible to the echinocandins, but localized spikes in resistance are problematic and warrant attention (15, 16). Finally, infections due to rare *Candida* spp., many of which are intrinsically resistant, are increasingly being described (1, 9). Of these, *C. auris* is a major threat globally due to its pervasive nature and frequent resistance to one or more classes of antifungal agents (17).

Global and local surveillance studies are important to monitor antifungal resistance. Global surveillance is particularly good at detecting and defining emerging threats, while local studies provide useful data to inform empirical therapy and aid antifungal stewardship efforts (8).

Increased access to whole-genome sequencing (WGS) provides an additional opportunity to characterize the genetic mechanisms of antifungal resistance acquired by resistant isolates identified in antifungal surveillance studies. Echinocandin resistance in *Candida* spp. is largely caused by mutations in the 2 hot-spot regions of the genes encoding the catalytic subunit of 1,3-beta-glucan synthase, required for cell wall growth. These mutations render 1,3-beta-glucan synthase impervious to echinocandin activity (7, 18, 19). The genetic alterations causing azole resistance are more difficult to define, since it can involve multiple possible mechanisms affecting ergosterol synthesis and/or augmenting drug efflux pump activity (7). Understanding the mechanisms of resistance encountered in resistant clinical isolates can aid drug discovery and therapeutic guidelines.

In this study, we describe the epidemiology and antifungal susceptibilities of 4,715 clinical yeast isolates, including *Candida* spp., as well as *Cryptococcus neoformans*, *Trichosporon asahii*, *Saccharomyces cerevisiae*, and *Rhodotorula mucilaginosa*, recovered from Ontario patients from 2014 to 2018. Additionally, we used WGS analysis to investigate susceptible-turned-resistant (within a 6-month time frame) pairs of isolates obtained from individual patients to confirm instances of acquired resistance and to elucidate the genetic mechanisms underlying the resistant phenotypes. This study complements other recent Canadian (20) and North American (8, 21) data sets to describe antifungal resistance in Canada's most populous province.

RESULTS

From 2014 to 2018, a total of 5,171 clinical yeast isolates were evaluated for antifungal susceptibility. A data set of 4,715 isolates representing the first isolate of a species received from a patient and submitted to Public Health Ontario (PHO) for antifungal susceptibility testing (AFST) within a 6-month period (selected, for the purposes of this paper, to define an infection episode) was used for the statistics described below. Figure 1a and b show the range of yeast species isolated from the specimens, with *C. albicans* isolated most frequently (43.0%), followed by *C. glabrata* (21.1%), *C. parapsilosis* (15.0%), and *C. tropicalis* (6.2%). The relative species distributions of *C. albicans*, *C. glabrata*, and *C. parapsilosis* remained constant over the study time frame ($P = 0.14$, $P = 0.21$, and $P = 0.51$, respectively), but the proportion of *C. tropicalis* increased from 2014 (4.3%) to 2018 (7.0%) ($P = 0.004$). An additional 23 *Candida* spp. and 4 non-*Candida* yeasts, encompassing 11.6% and 3.1% of the isolates, respectively, were identified (Fig. 1a and b). The total numbers of yeast isolates submitted for AFST increased from 2014 to 2018, with an annual change in the number of yeast isolates per 100,000 population (22) of 7.4% (Fig. 1c). Most isolates were recovered from sterile sites (81%), with blood the most frequent specimen source (57%) (Fig. 2; see Table S1 in the supplemental material).

MICs based on broth microdilution (BMD) results for susceptibility to echinocandins,

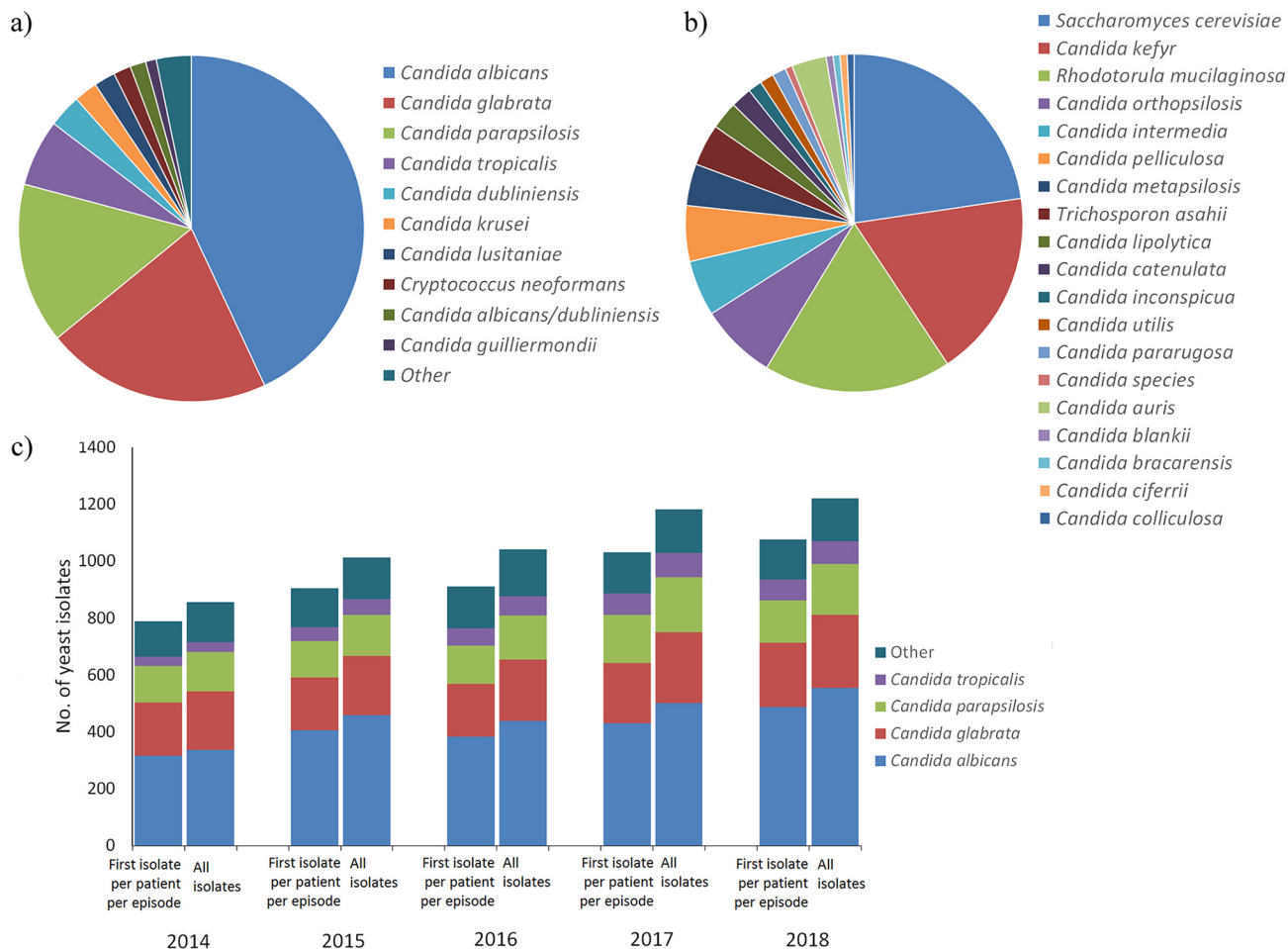


FIG 1 (a and b) Species distribution of common (a) and less common (b) yeast isolates from patient specimens representing the first isolate per patient per infection episode submitted for AFST in Ontario. (c) Species distribution per year from 2014 to 2018, including all isolates and the first isolate per patient per infection episode.

azoles, and amphotericin B are summarized in Table 1 for the more commonly encountered yeast species, i.e., *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. dubliniensis*, *C. krusei*, *C. lusitanae*, *C. neoformans*, and *C. guilliermondii*. Where CLSI breakpoints or Sensititre YeastOne (ThermoFisher, Waltham, MA) epidemiological cut-

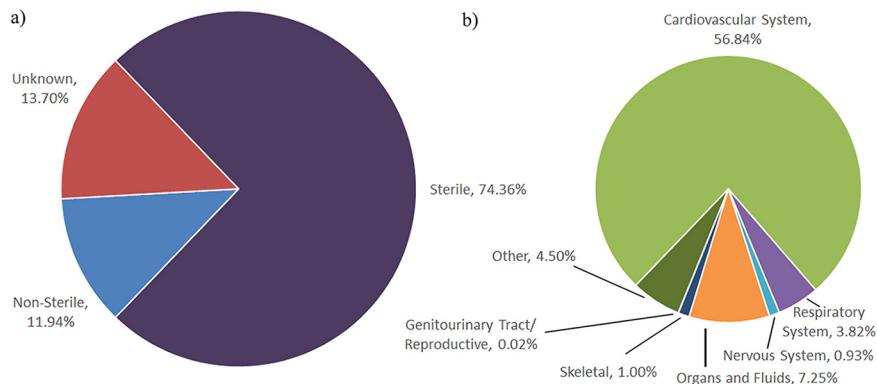


FIG 2 Specimen sources of yeast isolates representing the first isolate per patient per infection episode submitted for AFST in Ontario. (a) Distribution of sterile, nonsterile, and unknown specimens. (b) Distribution of specimens categorized into major organ systems.

TABLE 1 Activities of antifungal drugs against common yeast species according to CLSI clinical breakpoints (23) and/or recently determined Sensititre YeastOne ECVs (24, 25)

Organism, no. of isolates tested, and antifungal agent	Breakpoints (S, I, R) ^a ($\mu\text{g/ml}$)	ECV ($\mu\text{g/ml}$)	Range ($\mu\text{g/ml}$)	MIC ₅₀ ($\mu\text{g/ml}$)	MIC ₉₀ ($\mu\text{g/ml}$)	% of isolates ^b			
						CLSI method		ECV	
						S	R	WT	NWT
<i>C. albicans</i> , n = 2,029									
Fluconazole	≤ 2 , SDD = 4, ≥ 8	0.5	0.12 to >256	0.5	0.5	98	1	97	3
Voriconazole	≤ 0.12 , 0.25–0.5, ≥ 1	0.03	0.008 to >8	0.008	0.015	98	1	93	7
Posaconazole		0.06	0.008 to >8	0.03	0.03			98	2
Itraconazole			0.008 to 16	0.06	0.06			98	2
Amphotericin B		2	0.12 to 2	0.5	1				
Caspofungin	≤ 0.25 , 0.5, ≥ 1		0.008 to 8	0.06	0.06	100	0	100	0
Micafungin	≤ 0.25 , 0.5, ≥ 1	0.03	0.008 to 2	0.008	0.015	100	0	100	0
Anidulafungin	≤ 0.25 , 0.5, ≥ 1	0.12	0.015 to 1	0.015	0.06	100	0	100	0
<i>C. glabrata</i> , n = 994									
Fluconazole	SDD ≤ 32 , ≥ 64	8	0.12 to >256	16	32	91 ^c	9	93	7
Voriconazole		0.25	0.015 to 8	0.5	1			96	4
Posaconazole		1	0.008 to >8	1	2			92	8
Itraconazole		4	0.015 to >16	0.5	1			93	7
Amphotericin B		2	0.12 to 2	1	1				
Caspofungin	≤ 0.12 , 0.25, ≥ 0.5		0.008 to 8	0.06	0.12	93	1	99	1
Micafungin	≤ 0.06 , 0.12, ≥ 0.25	0.03	0.008 to 4	0.015	0.015	99	1	99	1
Anidulafungin	≤ 0.12 , 0.25, ≥ 0.5	0.25	0.015 to 2	0.03	0.06	99	1	99	1
<i>C. parapsilosis</i> , n = 708									
Fluconazole	≤ 2 , SDD = 4, ≥ 8	1	0.12 to 128	0.5	4	83	9	83 ^d	17 ^d
Voriconazole	≤ 0.12 , 0.25–0.5, ≥ 1	0.03	0.008 to 2	0.015	0.06	99	0	85 ^d	15 ^d
Posaconazole		0.25	0.008 to 0.25	0.03	0.06			100 ^d	0 ^d
Itraconazole		0.5	0.015 to 0.5	0.06	0.12			100 ^d	0 ^d
Amphotericin B		1	0.12 to 2	0.5	0.5				
Caspofungin	≤ 2 , 4, ≥ 8		0.06 to 1	0.5	1	100	0	100 ^e	0 ^e
Micafungin	≤ 2 , 4, ≥ 8	4	0.03 to 4	1	2	100	0	100 ^e	0 ^e
Anidulafungin	≤ 2 , 4, ≥ 8	8	0.03 to 4	1	2	100	0	100 ^e	0 ^e
<i>C. tropicalis</i> , n = 294									
Fluconazole	≤ 2 , SDD = 4, ≥ 8	1	0.25 to >256	2	8	79	12	88	12
Voriconazole	≤ 0.12 , 0.25–0.5, ≥ 1	0.12	0.008 to >8	0.12	0.5	61	8	92	8
Posaconazole		0.12	0.03 to >8	0.25	0.5			97	3
Itraconazole		0.5	0.03 to 16	0.25	0.5			94	6
Amphotericin B		2	0.25 to 2	1	1				
Caspofungin	≤ 0.25 , 0.5, ≥ 1		0.008 to 2	0.06	0.12	100	0	100	0
Micafungin	≤ 0.25 , 0.5, ≥ 1	0.06	0.008 to 0.06	0.03	0.03	100	0	100	0
Anidulafungin	≤ 0.25 , 0.5, ≥ 1	0.12	0.015 to 0.25	0.06	0.12	100	0	100	0
<i>C. dubliniensis</i> , n = 145									
Fluconazole		0.5	0.12 to 1	0.25	0.5			100	0
Voriconazole			0.008 to 0.03	0.008	0.008			99	1
Posaconazole		0.125	0.008 to 0.12	0.03	0.06			100	0
Itraconazole		0.25	0.015 to 0.12	0.03	0.06			100	0
Amphotericin B		0.5	0.12 to 1	0.5	0.5				
Caspofungin			0.015 to 2	0.06	0.12			99	1
Micafungin		0.12	0.008 to 1	0.03	0.03			99	1
Anidulafungin		0.12	0.015 to 0.12	0.12	0.12			100	0
<i>C. krusei</i> , n = 103									
Fluconazole			0.25 to 128	32	64			100	0
Voriconazole	≤ 0.5 , 1, ≥ 2	0.5	0.008 to 8	0.25	0.5	92	4	96	4
Posaconazole		0.5	0.03 to 1	0.25	0.5			100	0
Itraconazole		1	0.06 to 1	0.25	0.5			100	0
Amphotericin B		2	0.25 to 2	1	1				
Caspofungin	≤ 0.25 , 0.5, ≥ 1		0.03 to 2	0.25	0.5	85	1	99	1
Micafungin	≤ 0.25 , 0.5, ≥ 1	0.25	0.008 to 1	0.12	0.12	99	1	99	1
Anidulafungin	≤ 0.25 , 0.5, ≥ 1	0.25	0.015 to 0.5	0.06	0.06	99	0	99	1

(Continued on next page)

TABLE 1 (Continued)

Organism, no. of isolates tested, and antifungal agent	Breakpoints (S, I, R) ^a ($\mu\text{g/ml}$)	ECV ($\mu\text{g/ml}$)	Range ($\mu\text{g/ml}$)	MIC ₅₀ ($\mu\text{g/ml}$)	MIC ₉₀ ($\mu\text{g/ml}$)	% of isolates ^b				
						CLSI method		ECV		
						S	R	WT	NWT	
<i>C. lusitanae</i> , n = 94										
Fluconazole		1	0.12 to 32	0.5	2			98	2	
Voriconazole			0.008 to 0.25	0.008	0.03			95	5	
Posaconazole		0.06	0.008 to 0.25	0.03	0.06			98	2	
Itraconazole		1	0.015 to 0.5	0.06	0.12			100	0	
Amphotericin B		2	0.12 to 8	0.5	0.5					
Caspofungin		1	0.015 to 8	0.25	0.5			99	1	
Micafungin		0.5	0.008 to 8	0.06	0.12			98	2	
Anidulafungin		1	0.015 to 2	0.12	0.25			98	2	
<i>C. neoformans</i> , n = 77										
Fluconazole		8	0.5 to 8	2	4					
Voriconazole		0.25	0.008 to 0.06	0.03	0.06					
Posaconazole		0.25	0.008 to 0.25	0.06	0.12					
Itraconazole		0.25	0.015 to 0.12	0.03	0.06					
Amphotericin B		0.5	0.12 to 1	0.5	1					
Caspofungin			8	8	8					
Micafungin			4 to >8	8	8					
Anidulafungin			2 to >8	8	8					
<i>C. guilliermondii</i> , n = 47										
Fluconazole		8	0.12 to 32	2	8			98	2	
Voriconazole			0.008 to 1	0.06	0.25			98	2	
Posaconazole		0.5	0.03 to 1	0.12	0.5			100	0	
Itraconazole		2	0.06 to 2	0.25	0.5			98	2	
Amphotericin B		2	0.12 to 2	0.25	0.5					
Caspofungin	$\leq 2, 4, \geq 8$		0.06 to 1	0.25	0.5	100	0	100	0	
Micafungin	$\leq 2, 4, \geq 8$	2	0.06 to 1	0.5	1	100	0	100	0	
Anidulafungin	$\leq 2, 4, \geq 8$	8	0.12 to 2	1	2	100	0	100	0	

^aS, susceptible; I, intermediate; R, resistant.

^bIsolates described are the first isolate tested per patient per infection episode, 2014 to 2018. WT, wild type; NWT, non-wild type.

^cSDD, susceptible dose dependent.

^dECVs for fluconazole, voriconazole, posaconazole, and itraconazole are for *C. parapsilosis sensu stricto*.

^eECVs for caspofungin, micafungin, and anidulafungin are for *C. parapsilosis* species complex.

off values (ECVs) exist, the percent susceptible and resistant or wild-type and non-wild type were calculated (23–25).

When the first isolates submitted for AFST per patient per infection episode were examined, 98% of *C. albicans* isolates were susceptible to fluconazole and voriconazole; however, resistance to azoles was noted among non-*albicans* species. Nine percent of *C. glabrata* isolates were resistant to fluconazole. Similarly, 9% of *C. parapsilosis* isolates were resistant to fluconazole, and 83% were susceptible to the drug (8% were classified as susceptible dose dependent [SDD]) (Table 1). Azole resistance was more pronounced for *C. tropicalis* isolates, with 12% resistant (79% susceptible; 9% SDD) to fluconazole and 8% resistant (61% susceptible; 31% SDD) to voriconazole (Table 1). Caspofungin, micafungin, and anidulafungin exhibited good activity against *Candida* spp. Resistance rates for the echinocandins ranged from 0% to 1% for *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, *C. krusei*, and *C. guilliermondii*; of note, 7% of *C. glabrata* isolates and 15% of *C. krusei* isolates were nonsusceptible to caspofungin (Table 1). From 2014 to 2018 no statistically significant trends were detected pertaining to an increase or decrease in the percentage of the commonly isolated *Candida* spp. susceptible to various antifungal agents (Table 2). Isolates that were nonsusceptible to azoles or echinocandins were derived from a broad range of specimens (see Table S2 in the supplemental material) but were predominately bloodstream isolates of *C. parapsilosis*, *C. tropicalis* (azoles), and *C. glabrata* (echinocandins).

ECVs for use with Sensititre YeastOne panels for the detection of resistance among

TABLE 2 Percentages of *Candida* spp. susceptible to various antifungals for which CLSI breakpoints exist, 2014 to 2018

Organism and antifungal agent	% susceptible ^a					Cochran-Armitage P value ^b
	2014	2015	2016	2017	2018	
<i>C. albicans</i>						
Fluconazole	98	98	98	98	98	0.792
Voriconazole	98	99	98	99	98	0.966
Caspofungin	100	100	100	100	99	0.122
Micafungin	100	100	100	100	99	0.085
Anidulafungin	100	100	100	100	100	0.192
<i>C. glabrata</i>						
Caspofungin	99	87	95	93	92	0.206
Micafungin	99	99	98	99	100	0.659
Anidulafungin	99	99	98	99	100	0.825
<i>C. parapsilosis</i>						
Fluconazole	90	81	79	82	83	0.254
Voriconazole	98	98	99	99	99	0.345
Caspofungin	100	100	100	100	100	NA
Micafungin	100	99	99	100	100	0.533
Anidulafungin	100	99	100	100	100	0.425
<i>C. tropicalis</i>						
Fluconazole	76	77	85	75	79	0.963
Voriconazole	71	50	66	57	64	0.998
Caspofungin	100	100	100	100	99	0.220
Micafungin	100	100	100	100	100	NA
Anidulafungin	100	100	100	100	100	NA
<i>C. krusei</i>						
Fluconazole	95	95	86	95	89	0.584
Caspofungin	100	89	82	68	89	0.083
Micafungin	100	95	100	100	100	0.476
Anidulafungin	100	95	100	100	100	0.476
<i>C. guilliermondii</i>						
Caspofungin	100	100	100	100	100	NA
Micafungin	100	100	100	100	100	NA
Anidulafungin	100	100	100	100	100	NA

^aThe numbers represent the first isolate of each species per patient per infection episode.

^bNA, not applicable.

Candida spp. to triazoles (25) and echinocandins (24) have recently been determined. Using these values, a higher percentage (>10%) of isolates with non-wild-type voriconazole MICs were noted for *C. parapsilosis*. Likewise, >10% of *C. parapsilosis* and *C. tropicalis* isolates had non-wild-type fluconazole MICs (Table 1).

From 2014 to 2018, antimicrobial susceptibility testing was performed on 145 isolates of 23 less common *Candida* spp. and non-*Candida* yeasts representing the first isolates submitted for AFST per patient per infection episode. For species with ≥ 3 isolates, MIC distributions showing the number of isolates at each MIC for each species-drug combination are displayed in Table 3. As previously suggested, combining these data with similarly derived data from other studies would provide a more robust description of the MIC profiles of these rarely encountered species (21).

Among the rarely encountered *Candida* spp., elevated fluconazole MICs (>4 $\mu\text{g/ml}$) were noted for isolates of *C. orthopsilosis* ($n = 1/10$), *C. pelliculosa* ($n = 1/8$), *C. lipolytica* ($n = 1/4$) (Table 3), *C. inconspicua* ($n = 2/2$), *C. utilis* ($n = 2/2$), *C. pararugosa* ($n = 1/2$), *C. blankii* ($n = 1/1$), *C. bracarensis* ($n = 1/1$), *C. ciferrii* ($n = 1/1$), and *C. magnoliae* ($n = 1/1$) (data not shown). Voriconazole and posaconazole MICs were typically <1 $\mu\text{g/ml}$, except for 1 isolate each of *C. orthopsilosis* ($n = 1/10$), *C. pelliculosa* ($n = 1/8$), *C. lipolytica* ($n = 1/4$) (Table 3), *C. utilis* ($n = 1/2$), *C. blankii* ($n = 1/1$), *C. bracarensis* ($n = 1/1$), and *C. magnoliae* ($n = 1/1$) (data not shown). MICs of the echinocandins among the rarely

TABLE 3 Activities of antifungal drugs against uncommon yeast species with ≥3 isolates

Organism, no. of isolates tested, and antifungal agent	No. of isolates ^a at MIC (μg/ml) of:															MIC ₅₀ (μg/ml)	MIC ₉₀ (μg/ml)	
	≤0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128			>128
<i>S. cerevisiae</i> , n = 34																		
Fluconazole							6	3	9	6	4	4	1		1		2	16
Voriconazole		1	10	12	4	3	3		1								0.06	0.5
Posaconazole				2	4	11	10	2	4		1						0.5	2
Itraconazole				3	8	13	5		2			3					0.25	2
Amphotericin B					2	8	18	6									0.5	1
Caspofungin			1	2	10	15	6										0.25	0.5
Micafungin			1	4	18	10	1										0.12	0.25
Anidulafungin		1	2	4	18	6	3										0.12	0.25
<i>C. kefir</i> , n = 27																		
Fluconazole					8	13	6										0.25	0.5
Voriconazole	21	6															0.008	0.015
Posaconazole	2	1	6	9	7	1	1										0.06	0.12
Itraconazole		2	5	16	2	2											0.06	0.12
Amphotericin B					1		2	17	7								1	2
Caspofungin		8	11	4	2		1		1								0.03	0.12
Micafungin		1	8	12	3	1				1	1						0.06	0.25
Anidulafungin		2	3	8	8	3	1	1	1								0.12	0.5
<i>R. mucilaginosa</i> , n = 27																		
Fluconazole					1							1	1	1	5	18	256	>256
Voriconazole	1			1	1	2	1	4	7	9	1						2	4
Posaconazole	1				2	2	2	10	7		3						1	8
Itraconazole		1		1	2	2	6	12				3					1	16
Amphotericin B					1	4	16	6									0.5	1
Caspofungin			1					1		1	21						8	>8
Micafungin			1					1		2	20						3	8
Anidulafungin								1	1	1	21						3	8
<i>C. orthopsilosis</i> , n = 10																		
Fluconazole						1	5	2	2				1				1	8
Voriconazole	1	2	5	2						1							0.03	0.5
Posaconazole		1	2	2	6												0.12	0.12
Itraconazole		1	1	2	6		1										0.12	0.25
Amphotericin B						2	8	1									0.5	1
Caspofungin					1	3	6	1									0.5	1
Micafungin						2	7	2									0.5	1
Anidulafungin						1	5	4	1								0.5	2
<i>C. intermedia</i> , n = 8																		
Fluconazole					2	3	2	1									0.25	1
Voriconazole	8																0.008	0.008
Posaconazole	5	3															0.008	0.015
Itraconazole		2	4	2													0.03	0.06
Amphotericin B					3	2	3										0.25	0.5
Caspofungin				6	1	1											0.06	0.25
Micafungin		2	6														0.03	0.03
Anidulafungin		3	3	1	1												0.03	0.12
<i>C. pelliculosa</i> , n = 8																		
Fluconazole									3	4	1						4	8
Voriconazole				1	6	1											0.12	0.25
Posaconazole					1	3	3	1									0.5	1
Itraconazole					3	4	1										0.25	0.5
Amphotericin B					1	2	4	1									0.5	1
Caspofungin		1	3	3		1											0.06	0.12
Micafungin	1	3	4														0.03	0.03
Anidulafungin		8															0.015	0.015

(Continued on next page)

TABLE 3 (Continued)

Organism, no. of isolates tested, and antifungal agent	No. of isolates ^a at MIC ($\mu\text{g/ml}$) of:															MIC ₅₀ ($\mu\text{g/ml}$)	MIC ₉₀ ($\mu\text{g/ml}$)	
	≤ 0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128			>128
<i>C. metapsilosis</i> , n = 6																		
Fluconazole							1	4		1							1	4
Voriconazole	1	4		1													0.015	0.06
Posaconazole		1	4	1													0.03	0.06
Itraconazole			3	2	1												0.06	0.12
Amphotericin B						1	5										0.5	0.5
Caspofungin					3	2	1										0.25	0.5
Micafungin						2	4										0.5	0.5
Anidulafungin				1	4		1										0.12	0.5
<i>T. asahii</i> , n = 6																		
Fluconazole										3	2	1					4	8
Voriconazole			1	3	2												0.06	0.12
Posaconazole						4	2										0.25	0.5
Itraconazole					1	5											0.25	0.25
Amphotericin B						1	5										0.5	0.5
Caspofungin													6				8	8
Micafungin													6				8	8
Anidulafungin													6				8	8
<i>C. auris</i> , n = 5																		
Fluconazole							1	3	1								1	2
Voriconazole	2	3															0.015	0.015
Posaconazole	1	4															0.015	0.015
Itraconazole		1	1	3													0.06	0.06
Amphotericin B							1	3	1								1	2
Caspofungin			1	1		3											0.25	0.25
Micafungin			2	3													0.03	0.06
Anidulafungin					5												0.12	0.12
<i>C. lipolytica</i> , n = 4																		
Fluconazole									2	1					1		4	>256
Voriconazole			2	1									1				0.06	8
Posaconazole					1	1	1						1				0.5	8
Itraconazole					3									1			0.12	16
Amphotericin B							2	2									1	1
Caspofungin				1			3										0.5	0.5
Micafungin	1						3										0.5	0.5
Anidulafungin		1			2	1											0.12	0.25
<i>C. catenulata</i> , n = 3																		
Fluconazole					1				1	1							2	4
Voriconazole		1	1		1												0.03	0.12
Posaconazole		1	2														0.03	0.03
Itraconazole		1	2														0.03	0.03
Amphotericin B						1	2										0.5	0.5
Caspofungin				1		1	1										0.25	0.5
Micafungin		1	2														0.03	0.03
Anidulafungin			1	2													0.06	0.5

^aIsolates represent the first species per patient per infection episode, 2014 to 2018.

encountered *Candida* spp. were typically quite low ($<0.5 \mu\text{g/ml}$), except for 2/27 isolates of *C. kefyr* and 5/10 isolates of *C. orthopsilosis* (Table 3). Notably, 5 isolates of *C. auris* were recovered from 2014 to 2018; however, unlike what has been typically reported previously (17), none of the MICs for the 3 main classes of antifungal drugs were elevated. Of note, these isolates all belonged to clade IV (South American) of *C. auris* as determined by WGS analysis (26).

Among the non-*Candida* yeasts representing first isolates submitted for AFST per patient per infection episode, MICs of the echinocandins were very high for all isolates of *C. neoformans*, *R. mucilaginosa*, and *T. asahii*, although they remained low ($\leq 0.5 \mu\text{g/ml}$) for *S. cerevisiae* (Tables 1 and 3). Fluconazole MICs were also elevated ($>4 \mu\text{g/ml}$)

among some isolates of *C. neoformans* ($n = 3/77$), *S. cerevisiae* ($n = 10/34$), and *T. asahii* ($n = 1/6$) and most *R. mucilaginosa* isolates ($n = 26/27$), although the MICs for voriconazole and posaconazole were typically low (Tables 1 and 3).

When we analyzed all the submitted isolates, we noted examples of 24 sets of sequential isolates of *C. albicans* ($n = 5$), *C. glabrata* ($n = 12$), *C. parapsilosis* ($n = 2$), *C. tropicalis* ($n = 4$), and *C. krusei* ($n = 1$) from single patients that demonstrated a susceptible-to-nonsusceptible shift in antifungal resistance within a 6-month period (considered to be a single infection episode) (Table 4). Whole-genome sequencing was performed on select sets of these isolates to determine if the isolates were the same strain and in an attempt to identify the molecular mechanisms of resistance. In total, we sequenced 2 pairs of *C. albicans* (SP0206/SP0369 and SP2512/SP2683) and 1 pair of *C. tropicalis* (SP4694/SP4785) isolates that demonstrated a shift from azole susceptibility to resistance. We also sequenced 3 pairs of *C. albicans* (SP1153/SP1274, SP1261/SP1350, and SP4920/SP5012), 5 pairs of *C. glabrata* (SP1533/SP1643, SP2320/SP2659, SP3417/SP3689, SP3046/SP3439, and SP2983/SP3003), and 1 pair of *C. tropicalis* (SP4433/SP4501) isolates where the MICs of one or more echinocandins shifted from susceptible to resistant (Table 4). The numbers of days between an initial susceptible isolate and a subsequent resistant isolate ranged from 10 to 127. For 11 of the pairs (SP0206/SP0369, SP2512/SP2683, SP1153/SP1274, SP1261/SP1350, SP4920/SP5012, SP1533/SP1643, SP2320/SP2659, SP3417/SP3689, SP3046/SP3439, SP4694/SP4785, and SP4433/SP4501), multilocus sequence-typing (MLST) analysis and whole-genome single-nucleotide polymorphism (SNP) maximum-parsimony (MP) phylogenetic analysis suggested a high degree of genetic relatedness between the initial susceptible and subsequent resistant isolates from the same patient; the MLST sequence types were the same, and isolate pairs clustered together in the MP trees (Table 4; see Fig. S1 in the supplemental material). For one pair of *C. glabrata* isolates (SP2982/SP3003), the MLST sequence types were different and the isolates did not cluster together in the whole-genome SNP MP tree (Table 4; see Fig. S1). The specimen source of the initial susceptible isolate of this pair was peritoneal fluid, while the subsequent resistant isolate was derived from blood 10 days later (Table 4).

Comparison of the pairs of initial susceptible and subsequent resistant isolates of *C. albicans* where echinocandin resistance was acquired (SP1153/SP1274, SP1261/SP1350, and SP4920/SP5052) revealed that all 3 strains acquired an S645P mutation in hot spot 1 of *GSC1* (Table 4). Similar comparisons among initial susceptible and subsequent resistant isolates of *C. glabrata* revealed 3 strains (SP2320/SP2659, SP3417/SP2689, and SP3046/SP3439) that demonstrated acquisition of S663P in hot spot 1 of *FKS2* and 1 resistant isolate (SP3003) with an isoleucine instead of phenylalanine at position 625 of hot spot 1 of *FKS1* compared to the *C. glabrata* reference strain, CBS138. In one set of *C. glabrata* isolates (SP1533/SP1643), there were no mutations detected in *FKS1*, *FKS2*, or *FKS3* (Table 4). Comparison of the single pair of susceptible and resistant isolates of *C. tropicalis* from the same patient revealed the acquisition of an S30P mutation in CTRG_04661 (Table 4), which aligns with amino acid position 663 of the hot spot 1 region of *C. albicans GSC1*.

Among pairs of isolates demonstrating a shift from azole susceptible to azole resistant, we examined the following genes: ergosterol production enzyme genes *ERG3* and *ERG11*; efflux pump genes *CDR1*, *CDR2*, *CDR4*, *CDR5*, *CDR11*, *MDR1*, *FLU1*, *SNQ2*, *TPO3*, and *TOR1*; and transcription factor genes *MMR1*, *Ndt80*, *Stb5*, *TAC1*, and *UPC2* (7, 21, 27) (see Table S3 in the supplemental material). In the first pair of *C. albicans* isolates with acquired azole resistance, no detectable missense mutations in these targets were identified in the resistant isolate SP0369 relative to the initial susceptible isolate SP0206 (see Table S3). Conversely, multiple mutations were noted between the resistant (SP2683) and susceptible (SP2512) isolates of the second pair of *C. albicans* isolates, most notably a homozygous A255T mutation in *ERG3* (see Table S3). Comparison of genome-wide copy number variation between azole-resistant *C. tropicalis* strain SP4785 and its diploid progenitor, SP4694, suggested that the estimated overall ploidy of supercontig 3.8 was ~ 4 (see Table S3 and Fig. S2 in the supplemental material), with

TABLE 4 Sets of sequential isolates from single patients that demonstrated a susceptible (S)-to-nonsusceptible (I or R) shift in antimicrobial resistance within a 6-month period, representing a single infection episode

Organism and patient ID	Specimen ID	Specimen source	Interval (no. of days)	FLU ^a		VORI ^a		CASP ^a		MICA ^a		ANI ^a		Molecular mechanism of resistance	
				MIC	Resistance	MIC	Resistance	MIC	Resistance	MIC	Resistance	MIC	Resistance		ST ^a
<i>C. albicans</i>															
PA0188	SP0206	Blood	0	0.5	S	≤0.008	S							918	Unknown
	SP0369	Pleural fluid	83	≥256	R	8	R							918	
PA2062	SP2512	Blood	0	2	S	0.015	S							726	ERG3: A255T
	SP2683	Blood	55	256	R	8	R							726	
PA0909	SP1153	Blood	0					0.06	S	0.015	S	≤0.015	S	3568	GSC1: S645P
	SP1274	Blood	55					>8	R	2	R	0.25	S	3568	
PA1070	SP1261	Blood	0					0.5	I	0.06	S	0.06	S	3570	GSC1: S645P
	SP1350	Peritoneal fluid	34					4	R	2	R	0.5	I	3570	
PA3104	SP4920	Blood	0					0.12	S	0.015	S	≤0.015	S	3569	GSC1: S645P
	SP5052	Blood	39					8	R	4	R	1	R	3569	
<i>C. glabrata</i>															
PA1293	SP1533	Abscess	0					0.06	S					10	None detected
	SP1643	Abdomen	35					0.5	R					10	
PA3825	SP4776	Blood	0					0.06	S						
	SP4784	Blood	2					0.25	I						
PA1823	SP2186	Blood	0					0.03	S						
	SP2198	Blood	5					0.25	I						
PA2095	SP2554	Urine	0					0.06	S						
	SP2585	Urine	9					0.25	I						
PA3539	SP4419	Pleural fluid	0					0.06	S						
	SP4650	Pleural fluid	80					0.25	I						
PA1563	SP1866	Peritoneal fluid	0							0.015	S				
	SP2187	Blood	115							0.12	I				
PA1188	SP1405	Blood	0					0.12	S			0.12	S		
	SP1475	Blood	26					0.25	I			0.25	I		
PA1921	SP2320	Blood	0					0.12	S	0.03	S	0.03	S	16	FKS2: S663P
	SP2659	Blood	118					8	R	2	R	2	R	16	
PA2117	SP2583	Blood	0					0.12	S	0.015	S	0.03	S		
	SP2672	Blood	28					1	R	0.5	R	1	R		
PA2320	SP3417	Blood	0					0.06	S	0.015	S	0.03	S	3	FKS2: S663P
	SP3689	Blood	75					8	R	8	R	2	R	3	
PA2472	SP3046	Blood	0					0.12	S	0.015	S	0.06	S	19	FKS2: S663P
	SP3439	Blood	127					0.25	I	0.12	I	0.5	R	19	
PA2419	SP2982	Peritoneal fluid	0					0.06	S	0.015	S	≤0.015	S	17	
	SP3003	Blood	10					0.5	R	0.12	I	0.25	I	3	FKS1: F625I
<i>C. parapsilosis</i>															
PA1947	SP2355	Wound	0											0.12	S
	SP2398	Other	17											0.25	I
PA2106	SP2568	Blood	0											0.06	S
	SP2675	Blood	35											0.25	I

(Continued on next page)

TABLE 4 (Continued)

Organism and patient ID	Specimen ID	Specimen source	Interval (no. of days)	FLU ^a		VORI ^a		CASP ^a		MICA ^a		ANI ^a		Molecular mechanism of resistance
				MIC	Resistance	MIC	Resistance	MIC	Resistance	MIC	Resistance	MIC	Resistance	
<i>C. tropicalis</i>														
PA3063	SP3823	Blood	0		0.12	S								
	SP3887	Blood	25		0.25	I								
PA3762	SP4694	Peritoneal fluid	0	1	S	S							975	Aneuploidy of supercontig 3.8 and increased copy no. of ERG11 and TAC1
	SP4785	Peritoneal fluid	28	32	R	R							975	
PA3550	SP4433	Blood	0				0.06	S	0.015	S	0.12	S	974	CTRG_04661: S30P
	SP4501	Blood	22				8	R	2	R	1	R	974	
<i>C. krusei</i>														
PA1488	SP1777	Blood	0				0.25	S						
	SP1811	Blood	15				0.5	I						

^aFLU, fluconazole; VORI, voriconazole; CASP, caspofungin; MICA, micafungin; ANI, anidulafungin; MIC, minimum inhibitory concentration; R, resistance; S, susceptibility; I, intermediate; ST, sequence type.

the ploidy of the regions containing the *ERG11* sterol 14-demethylase gene and the *TAC1* transcriptional-activator gene estimated at 6.0 and 6.2, respectively.

DISCUSSION

Surveillance of antifungal susceptibility/resistance among clinical yeast species is an important endeavor given reports of resistance acquisition during treatment, the clinical impact of a variety of uncommon yeasts that are refractory to antifungal agents (9), and the potential for nosocomial spread of resistant strains (28–30). While global surveillance can identify emerging threats, local monitoring is also useful, since it can provide geographically focused information to aid empirical treatment and inform antifungal stewardship work.

The current study describes susceptibility/resistance rates among *Candida* spp. and non-*Candida* yeast species received at the provincial reference laboratory in Ontario for antifungal susceptibility testing. As PHO performs the vast majority of AFST in the province and Ontario is Canada's most populous province, representing almost 40% of the country's population (22), these data have potential to be relevant nationally. Isolates were submitted from both hospital and community laboratories, with the majority cultured from blood and other sterile sites. Similar to other reports from North America, Europe, and Australia, the majority of the isolates were *C. albicans*, followed by *C. glabrata* and *C. parapsilosis* (8, 20, 31–35). In other geographic regions, including Latin America, Asia, South Africa, the Middle East, and North Africa, *C. glabrata* is less frequently isolated, with *C. parapsilosis* and/or *C. tropicalis* recovered more predominantly in the species distribution (36–40). The proportional species distribution remained fairly constant from 2014 to 2018, except for a significant increase in *C. tropicalis*. Other studies have also noted a shift toward non-*albicans* clinical yeast isolates (1, 11, 13, 20). The total number of yeast isolates submitted each year for AFST standardized to population size appears to be increasing, with an annual change of approximately 7.4%. This may signal an increasing prevalence of invasive yeast infections in Ontario, possibly due to an aging population and/or an increasing immunocompromised patient population, as has been noted previously (1), or in the face of reports of increased resistance rates in yeast, it may represent decreased confidence among clinicians to treat empirically, based on species assignment and the desire to have actual susceptibility results or MICs.

Among the set of first isolates per patient per infection episode, *C. albicans* isolates were largely susceptible to both azoles and echinocandins, as in other studies (8, 20, 21, 33, 36, 39). Resistance rates for *C. glabrata* to all echinocandins were around 1%, which is lower than those reported for other sets of North American isolates (8, 20, 21). Echinocandin resistance in *C. glabrata* is more common in North America than in Europe and is quite rare in Asia and Latin America (8, 21, 31, 41). Conversely, azole resistance among *C. parapsilosis* (9%) and *C. tropicalis* (12%) isolates was higher here than that observed in other North American studies (8, 20). Higher levels of azole resistance among *C. parapsilosis* and *C. tropicalis* have been observed in Europe and Asia, respectively (31, 34, 35, 39, 41, 42). Differences between antifungal susceptibility rates in Ontario and in the other North American studies may be due to a large foreign-born population in Ontario (~30%) (43), as well as frequent travel to and from a diverse collection of countries of origin compared to other populations studied. Resistance rates have remained stable in Ontario from 2014 to 2018.

In the data set of first isolates per patient per infection episode, the number of yeast isolates other than *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* was notable, with 11.6% identified as other *Candida* species and 3.1% as non-*Candida* yeasts (*C. neoformans*, *S. cerevisiae*, *T. asahii*, and *R. mucilaginosa*). Since many of these species exhibited elevated MICs for one or more azole or echinocandin drugs (9), antifungal susceptibility data for isolates of these rarely encountered species have been included in this study to contribute to the scarce literature. This can help inform treatment and can potentially be combined with similar data sets from other studies to provide a more robust description of MIC profiles (21). Among the non-*Candida* yeasts, the isolation of

5 *C. auris* isolates is noteworthy and unusual in the context of the global *C. auris* crisis (17) in that all 5 appeared to be susceptible to all 3 major classes of antifungal agents. All 5 isolates, though from individual patients over a span of several years, have been determined through WGS analysis to be members of the South American clade (IV) and also to be closely related to one another (26).

Within the data set, 24 pairs of isolates were identified where a patient's first isolate was susceptible to an antifungal agent while a subsequent isolate within 6 months, defined here as representing a single infection episode, was nonsusceptible. Of these, 12 pairs were investigated by WGS to determine if the isolates represented the same strain and, if so, to attempt to elucidate the potential causal molecular mechanism(s) of acquired resistance. MLST and whole-genome SNP MP phylogenetic analysis demonstrated that for 11 of the 12 pairs (5 *C. albicans*, 4 *C. glabrata*, and 2 *C. tropicalis*), the first susceptible isolate and the subsequent resistant isolate were the same strain, thus confirming that resistance was acquired by the strain via within-host evolution rather than a strain replacement event. In one case (PA2419), a patient had a set of *C. glabrata* strains isolated from peritoneal fluid (SP2982; susceptible) and blood (SP3003; echinocandin nonsusceptible) 10 days apart; however, the two isolates had different sequence types, indicating that they did not represent within-host evolution of resistance, but instead, the patient harbored 2 different strains of *C. glabrata*.

In 6 of 7 cases of acquired echinocandin resistance, nonsynonymous mutations resulting in missense variants in a 1,3-beta-glucan synthase protein were noted between the initial susceptible and subsequent resistant isolates. This mechanism of echinocandin resistance is well characterized, and the *GSC1* S645P and *FKS2* S663P mutations are frequently observed in echinocandin-resistant clinical isolates of *C. albicans* and *C. glabrata*, respectively (18, 19, 45, 46). One echinocandin-resistant isolate of *C. glabrata* (SP3003) had an F625I mutation in *FKS1* compared to the *C. glabrata* reference strain, CBS138. Although in this case (PA2419) we lacked a prior paired susceptible strain (see above) (Table 4), position 625 of *FKS1* is within hot spot 1 (19, 45), suggesting that it is the cause of echinocandin resistance in the isolate. For one susceptible-to-resistant *C. glabrata* isolate pair (SP1533/SP1643), no mutations were detected in the 1,3-beta-glucan synthase gene *FKS1*, *FKS2*, or *FKS3*. This has been noted in other studies, particularly with low-level resistance, as in this case (caspofungin MIC, 0.5 μ g/ml), and supports the suggestion of alternate mechanisms of echinocandin resistance yet to be described (47–50). Comparison of the initial susceptible and subsequent echinocandin-resistant isolates of *C. tropicalis* (SP4433/SP4501) revealed an S30P mutation in CTRG_04661, which aligns with known hot spot 1 position 663 in its ortholog, *GSC1*, in *C. albicans* and has previously been reported as a mechanism of echinocandin resistance in this organism (51, 52).

The cause of azole resistance is more difficult to elucidate, since it can be mediated by multiple mechanisms, including activation of a variety of efflux pumps due to mutations in regulatory elements or transcription factors and nonsynonymous mutations or upregulation of one of several genes involved in ergosterol synthesis (7). For 1 strain of *C. albicans* (SP0206/SP0369) that developed azole resistance, no nonsynonymous mutations were detected in candidate azole resistance genes despite extensive searching. Resistance in this strain is likely mediated by a mechanism not detectable by our analysis, i.e., gene upregulation. Although a variety of mutations were noted in the candidate azole resistance genes for the second pair of *C. albicans* susceptible-resistant isolates (SP2512/SP2683), the most significant was a homozygous A255T mutation in *ERG3*. Defective or missing *ERG3* renders *C. albicans* azole resistant (53), and mutations in *ERG3* have been noted in azole-resistant clinical isolates (45, 54), making this the likely cause of azole resistance acquired by *C. albicans* in this case. For *C. tropicalis* isolate pair SP4694/SP4785, which was investigated for the development of azole resistance, the most significant alteration detected and the likely cause of azole resistance acquisition was the increase in copy number variation of supercontig 3.8. While the exact chromosomal configuration was not determined, YMAP analysis suggested that the entire supercontig 3.8 was duplicated, progressing from diploid to a

ploidy of ~ 4 , with a partial section located on the left side progressing to a ploidy of ~ 6 , possibly through formation of an isochromosome (55). The ergosterol synthesis gene *ERG11* and *TAC1*, the transcriptional activator of *CDR1* and *CDR2*, are located on the left side of supercontig 3.8, suggesting that aneuploidy in the strain mediated azole resistance by *ERG11* and *TAC1* gene amplification. Although this is a previously undescribed resistance mechanism in *C. tropicalis*, it is a known mechanism of azole resistance in *C. albicans* (55), and point mutations and increased expression of *ERG11* have been previously described as mechanisms of azole resistance in *C. tropicalis* (56, 57).

Although this study examined a fairly large collection of clinical yeast isolates, we acknowledge several limitations of the study. The first limitation is our lack of clinical information on the patients from whom the yeasts were isolated. We do not know the patient setting (e.g., inpatient or outpatient) or treatment history. Knowledge of what antifungal agents the patients received would allow more meaningful analysis of our results but was unavailable to us. A second limitation is the fact that, although we performed much of our analysis using the first isolate submitted to our laboratory, this may not truly represent the patient's first isolate, as that may not have been forwarded to us for AFST. Similarly, this study does not capture yeast isolates for which no susceptibility testing was requested and the patients were managed empirically. Although we perform the majority of AFST for yeast in the province, some local laboratories do not forward their isolates to us and perform their own testing in house. Finally, no comparison was made between the CLSI broth microdilution and the Sensititre YeastOne methods, although previous studies have demonstrated good agreement between the methods (58–60), and CLSI breakpoints have previously been applied to Sensititre YeastOne MICs (61). Also, ECVs developed for *Candida* spp. with Sensititre YeastOne are typically within one 2-fold dilution of those determined by the CLSI reference method (24, 25), demonstrating close agreement. Further research is required to establish clinical breakpoints for use with the Sensititre YeastOne panels.

In conclusion, this study describes the species distribution, antifungal susceptibility patterns, and molecular mechanisms of resistance of clinical yeast isolates in Ontario, Canada's most populous province. Our data suggest that both the species distribution and the rates of resistance remained constant from 2014 to 2018, with the exception of a small but significant increase in the proportion of *C. tropicalis* among yeast isolates. Rates of resistance to all three classes of antifungal drugs remained relatively low in our population. We also demonstrated the utility of whole-genome sequencing to confirm cases of acquired resistance and to identify the molecular mechanisms associated with resistance. Ongoing testing and surveillance of invasive and recalcitrant yeast infections, as well as public health vigilance, are recommended given the global threat of increasing antifungal resistance (10) and the confirmed presence of *C. auris* among Ontario patients.

MATERIALS AND METHODS

Organisms and susceptibility testing. The Public Health Ontario Laboratory (PHOL) is the provincial reference microbiology laboratory for Canada's most populous province, encompassing 39% of the country's population (14.5 million of 37.6 million people in 2019) (22). PHOL performs the majority of AFST in Ontario for hospitals and community laboratories. ASFT is performed on request from the treating physician and is typically restricted to invasive isolates from normally sterile sites. Acceptance criteria are relaxed for immunocompromised individuals, patients in intensive care units (ICU), and those failing empirical therapy, in which cases isolates from nonsterile sites may also be tested. Antifungal susceptibility data were retrospectively collected for a total of 5,171 clinical yeast isolates, including *Candida* spp., *C. neoformans*, *T. asahii*, *S. cerevisiae*, and *R. mucilaginosa*, submitted to PHOL from 2014 to 2018 for AFST.

Yeast identification was performed by a combination of morphological; biochemical; matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), using a Bruker MALDI Biotyper (Bruker Daltonics, Billerica, MA); and ITS2 sequence analyses (62–64).

In vitro AFST for fluconazole, voriconazole, posaconazole, itraconazole, amphotericin B, caspofungin, micafungin, and anidulafungin was performed using the BMD-based Sensititre YeastOne Y09 panels (ThermoFisher, Waltham, MA), with MIC results read at 24 h for *Candida* spp. and up to 72 h for other yeast species, when adequate growth in the positive-control well was observed (65, 66). CLSI breakpoints were applied according to the guidelines of CLSI M60 (23). *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were routinely included as quality control organisms (23), with results within acceptable ranges.

The rate of increase of yeast isolates submitted for AFST from 2014 to 2018 was estimated as the slope of the line of the natural logarithm of the incidence rate per 100,000 population from 2014 to 2018. The Cochran-Armitage test was used to determine the statistical significance ($P < 0.05$) of temporal trends of proportional changes in species distribution and percent susceptibility. Analyses were performed in R v3.6.2 (67), using the DescTools package (68).

The initial data set of 5,171 clinical yeast isolates was culled to remove duplicate isolates of the same species received from the same patient within a 6-month period, with the assumption that they represented multiple isolates from single infection episodes. Specimen source distribution, species distribution, MIC statistics, susceptible/nonsusceptible percentages, and wild-type/non-wild-type percentages were calculated from the culled data set containing 4,715 isolates using CLSI MIC breakpoints and published Sensititre YeastOne ECVs (23–25).

The initial data set was also examined for sets of sequential isolates from single infection episodes for single patients that demonstrated a susceptible-to-nonsusceptible shift in antimicrobial resistance. These isolates were selected for further analysis, including WGS.

Whole-genome sequencing analysis. Whole-genome sequencing was performed on select sets of *C. albicans*, *C. glabrata*, and *C. tropicalis* isolates that represented pairs of sequential isolates obtained from the same patient less than 6 months apart, representing single infection episodes, where the AFST profile shifted from susceptible to resistant. Total genomic DNA prepared using a ZymoBiomix DNA Miniprep kit (Zymo Research, Irvine, CA, USA) was used as input for library preparation using a Nextera XT DNA Library Prep kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Sequencing was performed on a MiSeq using a MiSeq reagent kit v3 (Illumina).

Genome assembly and SNP calling were performed according to the GATK Best Practices (79–81). Briefly, paired-end reads of each isolate were mapped against its respective reference genome (*C. albicans* SC5314 haplotype A version A22, *C. glabrata* CBS138, or *C. tropicalis* MYA-3404) downloaded from the *Candida* Genome Database (69) using the Burrows-Wheeler alignment tool (BWA) version 0.7.17 (70) with the BWA-MEM algorithm and Picard tools v 2.9.0 (<http://broadinstitute.github.io/picard>). Indel realignment and base recalibration were performed with GATK v 3.8 using known indel and polymorphisms files (71) for *C. albicans* or indels and polymorphisms obtained from an initial round of HaplotypeCaller (44) for *C. glabrata* and *C. tropicalis*. HaplotypeCaller was employed in GVCF mode, followed by joint genotyping of all isolates of each species. SNPs were hard filtered based on the following parameters: QD, <2.0; FS, >60.0; MQ, <40.0; SOR, >3.0; MQRankSum, less than -12.5; and ReadPosRankSum, less than -8.0. The hard-filtering parameters for indels were as follows: QD, <2.0; FS, >200.0; SOR, >10.0; ReadPosRankSum, less than -20.0. For each isolate, SNPs and indels with read depths (DP) of <10 were filtered.

MLST profiles were generated by uploading genomes created using the GATK FastaAlternateReference-Maker tool (72–74; <https://pubmlst.org>). Maximum-parsimony phylogenetic analysis of whole-genome SNPs with 500 bootstrap replications was performed in Mega v 10.1.6 (75) following Clustal W (76) alignment. All positions containing gaps and missing data were eliminated (complete deletion option).

Finally, select genes known to be involved in azole or echinocandin resistance were examined to detect genotypic differences between isolate pairs from the same patient that demonstrated a shift from susceptible to resistant. The genes associated with azole resistance and their corresponding locus tags are listed in Table S3. The locus tags of the 1,3-beta-glucan synthase genes associated with echinocandin resistance were as follows: *C. albicans* GSC1 (CAALFM_C102420CA), GSL2 (CAALFM_CR00850CA), and GSL1 (CAALFM_C105600WA); *C. glabrata* FKS1 (CAGL0G01034g), FKS2 (CAGL0K04037g), and FKS3 (CAGL0M13827g); and *C. tropicalis* CTRG-04661, CTRG_04806, and CTRG_00996. SnpEff v 4.3t (77) was used to annotate the SNP differences to identify those that produced missense variants. Finally, genome maps of resistant strains were visualized using YMAP (78), where the corresponding susceptible strain was selected as the parental strain comparator in order to visualize genome-wide ploidy estimates, copy number variation, and loss of heterozygosity events.

This work was reviewed and approved by PHO's Research Review Board, as well as Research Ethics. A privacy impact assessment was also completed.

Accession number(s). Raw sequences of the isolates have been deposited in the NCBI BioProject database under accession number [PRJNA610214](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA610214).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

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