Candida Species From Eye Infections: Drug Susceptibility, Virulence Factors, and Molecular Characterization

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Purpose. To determine the type of *Candida* species in ocular infections and to investigate the relationship of antifungal susceptibility profile to virulence factors.

Methods. Fifty isolates of yeast-like fungi from patients with keratitis, endophthalmitis, and orbital cellulitis were identified by Vitek-2 compact system and DNA sequencing of ITS1-5.8S-ITS2 regions of the rRNA gene, followed by phylogenetic analysis for phenotypic and genotypic identification, respectively. Minimum inhibitory concentration of six antifungal drugs was determined by E test/microbroth dilution methods. Phenotypic and genotypic methods were used to determine the virulence factors.

Results. Phylogenetic analysis showed the clustering of all isolates into eight distinct groups with a major cluster formed *Candida parapsilosis* ($n = 21$), which was the most common species by both Vitek 2 and DNA sequencing. Using χ^2 test no significant difference was noted between the techniques except that Vitek 2 did not identify *C. viswanathii, C. orthopsilosis*, and two non-*Candida* genera. Of 43 tested *Candida* isolates high susceptibility to amphotericin B (39/43, 90.6%) and natamycin (43/43, 100%) was noted. While none of the isolates produced coagulase, all produced esterase and catalase. The potential to form biofilm was detected in 23/43 (53.4%) isolates. Distribution of virulence factors by heat map analysis showed difference in metabolic activity of biofilm producers from nonbiofilm producers.

Conclusions. Identified by Vitek 2 and DNA sequencing methods *C. parapsilosis* was the most common species associated with eye infections. Irrespective of the virulence factors elaborated, the *Candida* isolates were susceptible to commonly used antifungal drugs such as amphotericin B and natamycin.

Keywords: eye infection, *Candida*, virulence, antifungal susceptibility

Advanced medical treatment and increases in number of immunocompromised patients have contributed to increase in fungal infections worldwide. Use of long-term antibiotics and acquired immunodeficiency syndrome are other contributory factors. Apart from host immunity, climatic conditions and a predominance of fungi in tropical environments contribute to the prevalence of fungal infections including that of the eye. The cornea seems to be more susceptible to fungal infections compared with other parts of the eye in some regions of the world where fungal infections are common.[1,2] The prevalence of fungal keratitis in India varies from 7.3% to 44.8%.[3] In tropical parts of the world, filamentous fungi are the predominant causes of fungal keratitis and occur mostly following trauma. Yeast and other yeast-like fungi are less frequent causes of keratitis.[4] However, the prevalence of keratitis due to yeast-like fungi, notably *Candida* species, is reportedly higher in countries such as the United States and Mexico.[5–7] Very often candidiasis is associated with predisposing factors, such as excessive use of corticosteroids or antibiotics, surgical procedures, immunosuppression, systemic disease, or compromised ocular immunity. *Candida* species happen to be the most common cause of endogenous endophthalmitis.[8] In addition, they have been reported to cause postpenetrating keratoplasty endophthalmitis, ulcerative blepharitis, dacryocystitis, and so on.[8]

We recently reported the clinical and microbiological profile of 42 cases of ocular candidiasis wherein the Vitek 2 compact system (bioMérieux, Marcy l'Etoile, France) was used for the identification of the species of *Candida*.[9] Although commercial systems including Vitek 2 are very useful in clinical microbiology laboratories, their limitation in identification of uncommon yeasts is well known.[10] Identification of correct species and its correlation with their antifungal susceptibility is of medical importance.

Over the years, *Candida* strains have developed resistance to antifungal azoles and polyenes indicating the emergence of antifungal resistant strains.[11] In general, this resistance is acquired by the microorganisms mainly due to excessive use of antifungal drugs and virulence mechanisms developed by the microbes.[11,12] Biofilm is an important virulence factor responsible for antimicrobial resistance.[13] The other virulence mechanisms are involved in colonization, adhesion, invasion,

dissemination, and escape from host defense.[14,15] Not much is known about the biofilm formation and virulence factors in ocular candidiasis.

In the present study, ocular isolates of yeast-like fungi, initially characterized by the Vitek 2 compact system, were subjected to phylogenetic analysis based on Internal transcribed spacer region of rRNA genes (ITS) sequence. The isolates were screened for their potential to produce various virulence factors and the results were correlated with biofilm formation and antifungal susceptibility.

Materials and Methods

Isolation, Identification, and Antifungal Susceptibility Testing

The institutional review board of L. V. Prasad Eye Institute approved this study and the tenets of declaration of Helsinki were adhered to for conduct of the study. Fifty isolates of yeast-like fungi from various clinical samples from patients with eye infections were included in the study. Preserved isolates were revived and tested by Vitek 2 compact system using YST strips (bioMérieux) following manufacturer's instructions. The study isolates included those reported in our earlier publication.[9] Minimum inhibitory concentration (MIC) of six antifungal drugs (amphotericin B, itraconazole, voriconazole, fluconazole, caspofungin, and natamycin) against the 50 isolates were tested by agar diffusion E test (ET) using E strips (bioMérieux) or microbroth dilution method.[16] Clinical and Laboratory Standards Institute (CLSI) (M27-S4) interpretative guidelines for MIC breakpoints were followed to classify the isolates as susceptible (S), susceptible dose dependent (SDD), and resistant (R).[17] However, for the purpose of analysis, SDD isolates were clubbed with susceptible isolates. Breakpoints for itraconazole and natamycin (not available in CLSI guidelines) were noted as per published literature.[9]

DNA Isolation, Sequencing, and Phylogenetic Analysis

DNA was extracted from all isolates using QIAamp DNA kit (Qiagen, Hilden, Germany) and ITS1-5.8S-ITS2 regions of the rRNA gene was amplified by PCR as described earlier and the purified PCR products were sequenced using same primers.[18] Forward and reverse sequences of ITS region of each strain were assembled using SeqMan Pro (DNAStar, Inc., Madison, WI, USA) and consensus was obtained. Consequently, the identification of phylogenetic neighbors and the calculation of pairwise similarities were achieved through the NCBI BLASTn 2.2.26 program.[19] Phylogenetic tree construction using MEGA6 software was also carried out to ensure the genuineness in the identification.[20]

Phenotypic Characterization of Virulence Factors

All isolates were tested for a variety of virulence factors. Phenotypic methods were used to study the production of esterase, phospholipase, proteinase, coagulase, catalase, hemolysins,[21] and molecular detection of virulence genes, such as hyphal wall protein 1(*HWP*1), agglutinin like sequence1 (*ALS*1), alpha integrine like sequence 1 (*INT*1), proteinase 1 (*SAP*1), and phospholipase B 1 (*PLB*1) was done by PCR.[22] The potential to form biofilm was assessed using a microtiter 96-well tissue culture plate (TCP) method using a previously described method.[23] As described by the authors a cut-off value of OD 0.35 at 595 nm was arrived at with reference to the medium control and was set to determine biofilm positive isolates.

Statistical Analysis

Wherever applicable all comparisons were evaluated using χ^2 test for proportions and homogeneity and a P value of less than 0.05 was considered significant.

Results

Fifty isolates of yeast-like fungi from various clinical samples (corneal scrapings: 38, vitreous/subretinal fluid: 11, pus: 1) from patients with eye infections were processed as per the protocol described previously.[24] The patients were seen, investigated and treated at L. V. Prasad Eye Institute between September 2010 and May 2015. The isolates were preserved on Sabouraud dextrose agar slopes at room temperature and tested later. BLAST analysis of ITS region of the sequences of all 50 isolates showed 98% to 100% similarity with six species of *Candida* and one species each of *Meyerozyma* and *Cyberlindnera*. Phylogenetic analysis showed the clustering of all isolates into eight distinct groups (Fig. 1). A major cluster was formed by *Candida parapsilosis* ($n = 21$) followed by *Candida viswanathii* ($n = 9$) and *Candida albicans* ($n = 7$). *Candida glabrata* ($n = 4$), *Meyerozyma caribbica* ($n = 4$), *Cyberlindnera fabianii* ($n = 3$), *Candida tropicalis* ($n = 1$), and *Candida orthopsilosis* ($n = 1$) formed the minor groups. Sequences of all isolates were submitted to Genbank (KX090437-41, KT191106-26, KU739406-17, KU752208-13, KT191104-05, KT191127-28, KU950828, KU752214).

Table 1 shows DNA sequence based species wise distribution of all patients along with the brief demographics, follow-up period, and average visual acuity at presentation and outcome. Apart from an overall male preponderance, the table shows poor visual outcome in most patients with marginally better outcome in *C. parapsilosis* and *C. glabrata* infections.

Of the 50 isolates included in the study, the most common species was *C. parapsilosis* followed by *C. albicans* by both Vitek 2 and DNA sequence identification (Fig. 2a). While sequencing identified all isolates, two isolates were unidentified by the Vitek 2 system and were identified as *C. viswanathii* by sequencing. In addition, only through sequencing, nine *C. viswanathii* and one *C. orthosilopsis* species were identified. Also, one isolate, labeled as *Cryptococcus laurentii* through the Vitek 2 system, was identified as *Candida viswanathii* by sequencing. Similarly, seven isolates were placed in different genera *viz*., *Cyberlindnera fabianii*-3 and *Meyerozyma caribbica*-4 by sequencing, while they were labeled as several other species of *Candida* by Vitek 2 system, such as *C. utilis*-2, *C. famata*-2, *C. albicans*-1, and *C. guilliermondii*-2. A comparison of the two techniques of yeast identification is shown in Table 2. Larger number of isolates showed similarity in identification by both systems than dissimilarity (similar: 28, dissimilar: 20). A correlation of confidence in identification by Vitek 2 and sequence type was analyzed. Twelve of 28 isolates (42.8%, confidence interval [CI]: 26%–60%) with excellent confidence level in Vitek 2 matched in the identification by both systems. Among 20 nonmatching isolates there were three isolates (15%, CI: 5%–36%) that were labeled excellent and the difference was significant ($P = 0.04$). A mean probability of above 90% was seen in four major species of *Candida* such as *C. albicans, C. glabrata, C. famata,* and *C. parapsilosis*.

The antifungal susceptibility results were interpreted based on CLSI guidelines[17] and published literature. For the purpose

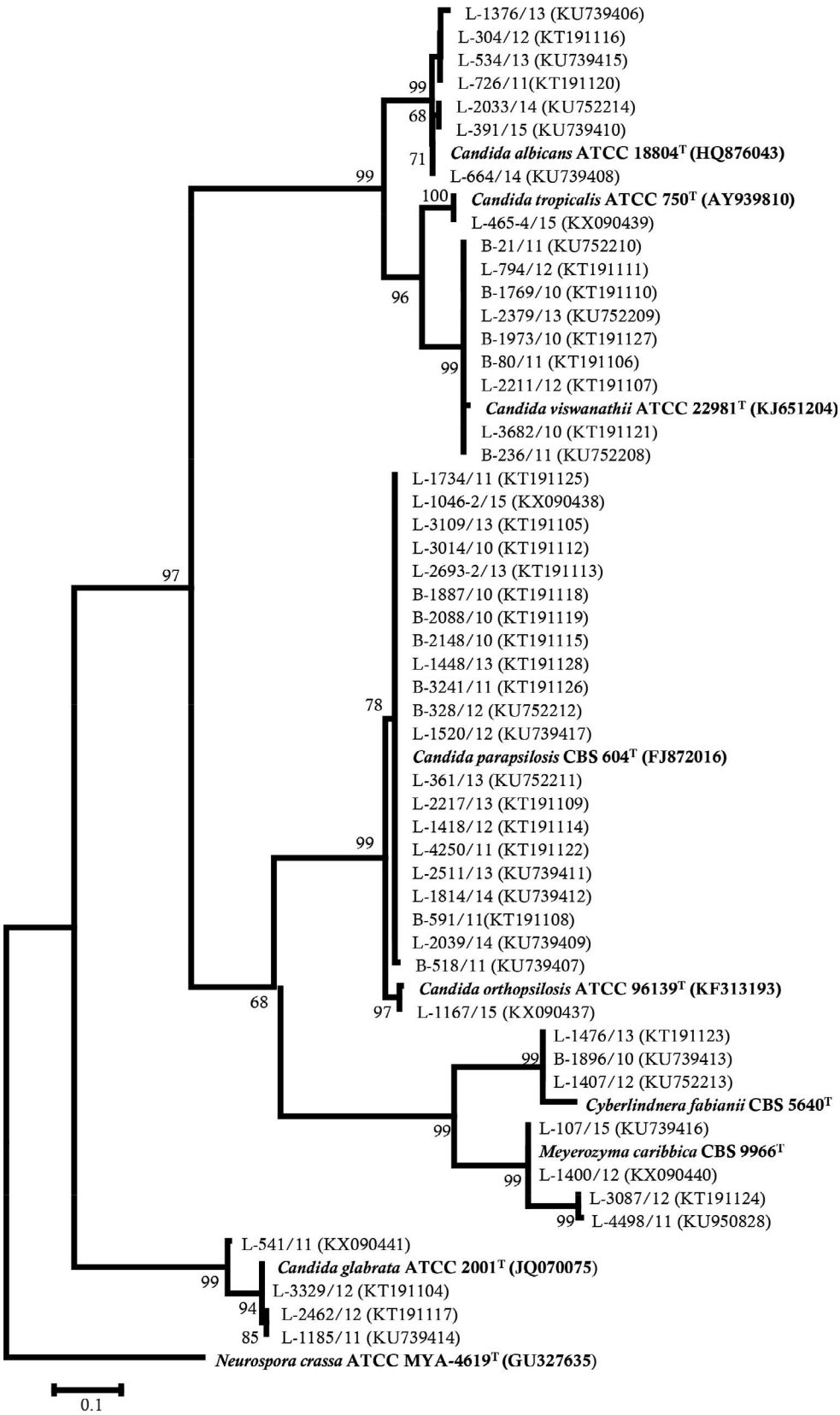

Figure 1. Phylogenetic tree based on ITS sequences showing the clustering of 50 clinical isolates with their closest relatives (shown in *bold*). The tree was constructed using the Maximium Likelihood method of MEGA6 software and rooted by *Neurospora crassa* as out-group. Bootstrap values (based on 1000 resamplings), of greater than 50% are shown at the nodes. The ITS sequences of all the type strains were retrieved from NCBI database, except *Meyerozyma caribbica* and *Cyberlindnera fabianii,* which were taken from Centraalbureau voor Schimmelcultures (CBS-KNAW) yeast nucleotide database. *Bar*: 10 nucleotides substitution per 100 nucleotides.

Table 1. Species-Wise Distribution (DNA-Sequence Based) of the Patients Along With the Brief Demographics, Follow-Up Period, and Visual Acuity at Presentation and Outcome

	Candida viswanathii	*Candida parapsilosis*	*Candida glabarta*	*Cyberlindnera fabianii*	*Meyerozyma caribbica*	*Candida albicans*	*Candida orthopsilosis*	*Candida tropicalis*
Keratitis	7	16	1	2	4	5	1	1
Endophthalmitis	2	5	3	1	0	1	0	0
Orbital cellulitis	0	0	0	0	0	1	0	0
Male	8	17	4	3	3	5	1	1
Female	1	4	0	0	1	2	0	0
Mean visual acuity at presentation, logMAR	2.2 ± 0.6	2.07 ± 0.9	1.8 ± 1.2	2.6 ± 1.7	2.45 ± 0.3	2.08 ± 0.9	1	2
Mean final best corrected visual acuity, logMAR	2.1 ± 0.8	1.62 ± 1.16	1.4 ± 0.9	2.6 ± 1.7	2.5 ± 0.5	2.17 ± 0.9	0.3	3
Mean follow-up period, d	88.22 ± 119.8	125 ± 150	17.7 ± 22.1	73.3 ± 103	97.5 ± 42.4	76 ± 56.9	7	40

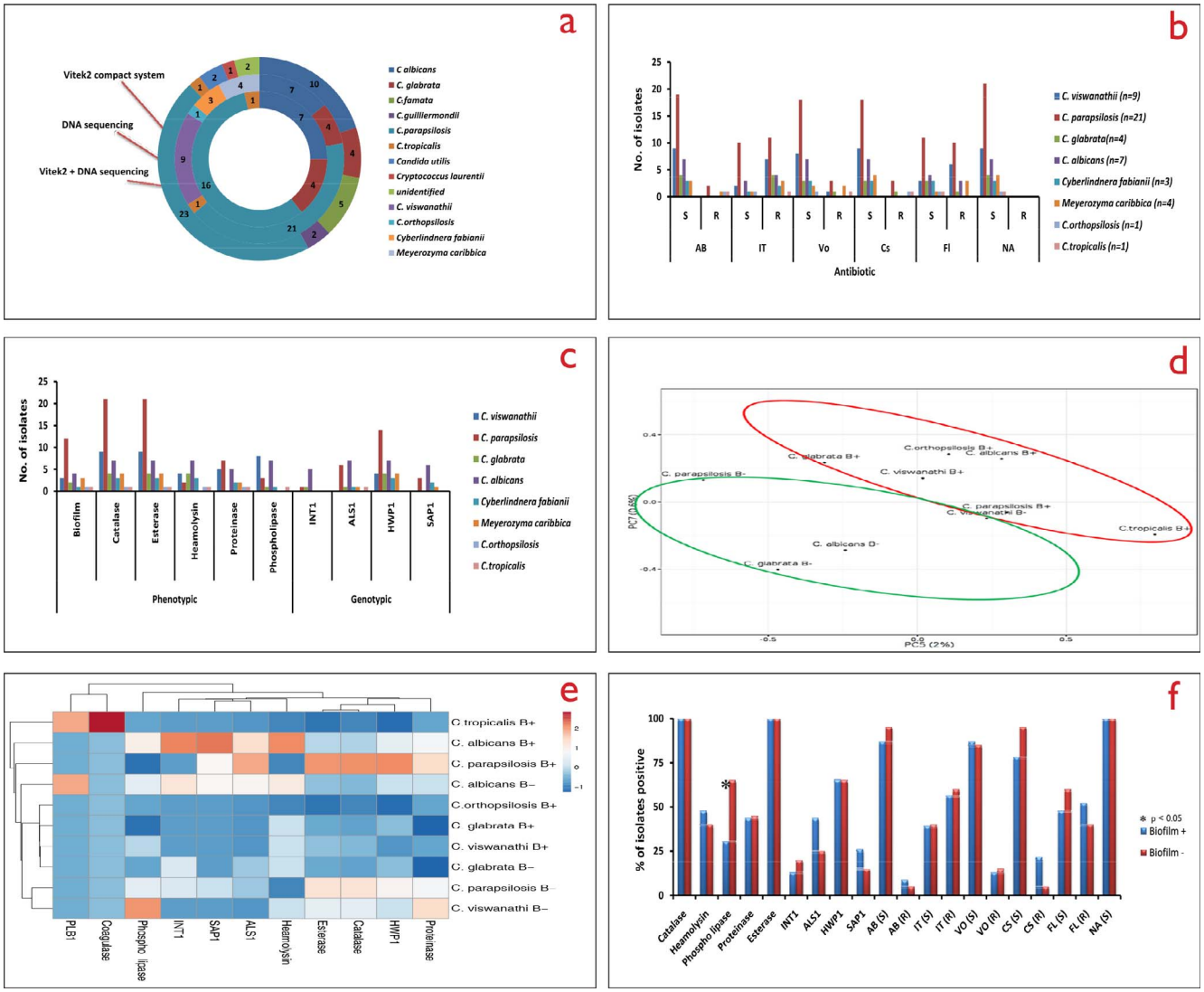

Figure 2. (**a**) Pie chart representation of comparison of identification of 50 *Candida* isolates by Vitek 2 compact system (*outer circle*) and DNA sequencing (*middle circle*). The *inner circle* represents similarity in species identification by both systems; (**b**) graphical representation of antifungal susceptibility of *Candida* species ($n = 50$) by E strip or drug dilution microtitre plate method. (**c**) Graphical representation of virulence factors produced by *Candida* isolates ($n = 43$); (**d**) PCA graphical representation showing correlation of biofilm with virulence factors. *Red circle* indicates the biofilm producers, which are separated from nonbiofilm producers in *green circle*. The two PCs chosen were 2% and 0.6% of the variation. B+ biofilm producer, B- non-biofilm producer; (**e**) Heat map representation of cluster analysis of biofilm in correlation with virulence factors. Heat map was generated using the correlation distance and Mcquitty linkage (similarity matrix). B+ biofilm producer, B- non-biofilm producer; (**f**) Correlation of antifungal susceptibility and virulence profile (genotypic and phenotypic) in biofilm-positive and -negative *Candida* isolates ($n = 43$). AB, amphotericin B; IT, itraconazole; VO, voriconazole; CS, caspofungin; FL, fluconazole; NA, natamycin; S, susceptible; R, resistant.

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Table 2. Comparison of Vitek 2 Compact System and DNA Sequencing in the Identification of Yeast-Like Fungi From Ocular Infections ($n = 50$)

	DNA Sequencing (%)	Vitek 2 Compact System (%)	*P* Value
C. albicans	7 (14)	10 (20)	0.4268
C. glabrata	4 (8)	4 (8)	1
C. famata	0	5 (10)	0.0225*
C. guilliermondii	0	2 (4)	0.1552
C. parapsilosis	21 (42)	23 (23)	0.6885
C. tropicalis	1 (2)	1 (2)	1
Candida utilis	0	2 (4)	0.155
Cryptococcus laurentii	0	1 (2)	0.3173
Unidentified	0	2 (4)	0.155
C. viswanathii	9 (18)	0	0.0018*
C. orthopsilosis	1 (2)	0	0.3173
Cyberlindnera fabianii	3 (6)	0	0.0802
Meyerozyma caribbica	4 (8)	0	0.0423*

* $P \leq 0.05$.

of analysis the susceptible dose dependent isolates were clubbed with susceptible. Figure 2b shows that the susceptibility to amphotericin B (45/50, 90%) and natamycin (50/50, 100%) was very high while it was variable to other antibiotics. A comparison of MIC (MIC_{50} and MIC_{90}) of six antifungal drugs tested against isolates of *Candida* spp. ($n = 43$), *Cyberlindnera fabianii* ($n = 3$), and *Meyerozyma caribbica* ($n = 4$) was made (Table 3). Significantly higher MIC_{50} and MIC_{90} were noted for caspofungin in *Candida* spp. compared with *Cyberlindnera fabianii* and *Meyerozyma caribbica* while higher MIC_{90} was seen for natamycin in *Candida* spp. compared with the other two. MIC_{50} of amphotericin B was comparable for all three groups of yeast and the difference in MIC_{50} and MIC_{90} was not significant.

The distribution of various virulence factors determined by phenotypic and genotypic methods is shown in (Fig. 2c). Esterase and catalase activity was found in all *Candida* isolates and all isolates were negative for coagulase production. Eleven of 21 (52.3%) *C. parapsilosis* were negative for all the three enzymes viz., hemolysin, phospholipase, and proteinase. A comparison of virulence factors in the two most common species – *C. parapsilosis* ($n = 21$) and *C. albicans* ($n = 7$) showed a significantly higher prevalence of virulence factors (β hemolysis, phospholipase, *INT*1, *ALS*1, and *SAP*1; $P < 0.001$) in *C. albicans*. However, there was no difference in the proportion of biofilm positive and biofilm negative isolates in these two species (57.1% in each) and all isolates (except one *C. parapsilosis*) in these two species were susceptible to amphotericin B and natamycin.

Analysis of biofilm formation in 43 *Candida* isolates (results of biofilm in 7 non-*Candida* isolates was excluded from analysis) showed that 23 were biofilm positive and 20 were biofilm negative. These isolates were also tested for various virulence factors by phenotypic (catalase, esterase, hemolysin, proteinase, phospholipase) and genotypic methods (*INT1, ASL1, HWP1, SAP1*). The virulence factors were analysed by principal component analysis (PCA) variables.[25] The analysis revealed a distinct segregation of the virulence factors into two major independent circles each representative of the biofilm and nonbiofilm formers (Fig. 2d). The two PCs (PC5 and PC7) were selected to provide the variation of data objects (2% and 0.6% of the variation) for convenient visualization and differentiation. Heat map[25] was constructed between biofilm producers and nonproducers (Fig. 2e). Cluster analysis using heat map was applied between two linkage groups to correlate distance and Mcquitty linkage (similarity matrix). The dendogram shows that *C. parapsilosis* and *C. viswanathii* nonbiofilm producers form a cluster independent of biofilm producers. This indicates a difference in their metabolic activity.

Table 3. Comparison of Minimum Inhibitory Concentration of Six Antifungal Drugs Tested Against Isolates of *Candida* spp., *Cyberlindnera fabianii*, and *Meyerozyma caribbica*

Species	Antibiotic	MIC_{50}, µg/mL	MIC_{90}, µg/mL	Range	Susceptible*	Resistant	% of Resistance	*P* Value, (MIC_{50} vs. MIC_{90})
Candida isolates ($n = 43$)	AB	0.19	1†	0.047–32	39	4	9.3	0.4576
	IT	0.75†	32†‡	0.094–32	18	25	58.1	<0.0001
	VO	0.125†	1†	0.016–128	37	6	14.0	0.4096
	CS	0.19	4‡	0.016–32	37	6	14.0	0.0578
	FL	6†‡	256†	0.5–256	13	20	46.5	<0.0001
	NA	4†‡	16†‡	2.0–16	43	0	0.0	0.0023
Cyberlindnera fabianii ($n = 3$)	AB	0.125	0.125	0.094–0.125	3	0	0.0	1
	IT	1	32	0.38 to >32	1	2	66.7	0.0202
	VO	0.064	0.19§	0.032–0.19	3	0	0.0	0.8155
	CS	0.094	1.5	0.064–1.5	3	0	0.0	0.2355
	FL	2§	12§	1.0–12.0	2	1	33.3	0.0345
	NA	4	8	2.0–8	3	0	0.0	0.2912
Meyerozyma caribbica ($n = 4$)	AB	0.19	1.5	0.032–1.5	3	1	25.0	0.2885
	IT	1	32	0.25 to >32	1	2	50.0	0.0043
	VO	0.5	32	0.064 to >32	2	2	50.0	0.0031
	CS	0.125	0.25	0.032–0.25	4	0	0.0	0.8501
	FL	12	256	1.5 to >256	1	3	75.0	0.0145
	NA	4	8	4–8.0	4	0	0.0	0.1266

$P \leq 0.05$ is significant.
* MIC breakpoints (µg/ mL) for susceptible: AB ≤ 1, IT ≤ 0.5, VO ≤ 0.75, CS ≤ 0.5, FL ≤ 4, NA ≤ 16.
† Significant for *Candida spp*. versus *M. caribbica*.
‡ Significant for *Candida* spp. versus *C. fabianii*.
§ Significant for *M. caribbica* versus *C. fabianii*.

Table 4. Clinical Details of Keratitis Patients With *Candida parapsilosis* Infection ($n = 16$)

Serial no.	Age, y/ Sex	Visual Acuity at Presentation, logMAR	Predisposing Factors	Topical Treatment	Systemic treatment	Duration of Follow Up, Days	Outcome	Visual Acuity at Last Follow Up, logMAR
1	36/M	2.7	Sand particles	Natamycin	Ketoconazole	16	TPK	1.3
2	34/M	2.4	Chemical injury	Amph B, Natamycin	Fluconazole	400	TPK	2.4
3	56/M	0.2	Wood particles	Natamycin	nil	4	LTFU	0.1
4	59/M	2.7	Unknown	nil	nil	4	LTFU	2.7
5	60/M	3	None	Natamycin	nil	135	Evisc	3
6	50/M	3	None	nil	nil	80	Evisc	3
7	52/M	0.8	Insect fall	Natamycin	nil	0	Resolved	0.5
8	29/F	0.8	None	Natamycin	nil	413	Resolved	0.1
9	64/M	LTFU	None	Fluconazole	Ketoconazole	0	LTFU	LTFU
10	21/F	0.9	None	Natamycin	Fluconazole	21	Resolved	0.1
11	39/M	0.5	None	Natamycin	Ketoconazole	114	Resolved	0.1
12	75/M	1.3	K-pro for vascularized cornea and PED following PK for fungal keratitits	Amph B, chloram, cefazolin, itra, vori, natamycin at different time points	nil	343	Resolved	1.1
13	67/M	3	Unknown	Amph B	Voriconazole	183	Phthisis	3
14	47/F	3	Unknown	Vori	nil	1	LTFU	3
15	29/M	2.7	Post-PK (for adherent leucoma) corneal infiltrate	Amph B	nil	461	Resolved	1.9
16	5/M	3	K-pro for SJS	Cefazolin, Cipro	nil	33	Corneal melt, LTFU	3

PK, penetrating keratoplasty; K-pro, Keratoprosthesis; Amph B, amphotericin B; Vori, voriconazole; Cipro, ciprofloxacin; Itra, itraconazole; Evisc, evisceration; PED, persistent epithelial defect.

Biofilm-positive *Candida* isolates ($n = 23$) showed marginally higher but statistically not significant resistance to antifungal agents than nonbiofilm producers ($n = 20$) (Fig. 2f). For instance, at a P value of 0.119, higher number of biofilm-positive isolates showed resistance to caspofungin (5/23, 21.7%) compared with biofilm negative isolates (1/20, 5%). Similarly, though not significant, the resistance to fluconazole was higher among biofilm forming isolates ($P = 0.43$). Presence of *ASL1* and *SAP1* gene was higher in biofilm positive isolates (10/23, 43.4%) than nonbiofilm isolates (5/20, 25%) indicating their role in attachment during biofilm formation and production of aspartyl proteinase in tissue invasion and defense mechanism.

A correlation of the predisposing factors, treatment received, and clinical outcome was done for patients infected with the most common species of *Candida*, that is, *C. parapsilosis* (Table 4) associated with keratitis ($n = 16$) and the rare species of *Cyberlindnera fabianii* and *Meyerozyma caribbica* (Table 5). Resolution of infection was seen in 4/16 (25%) patients, while five were lost to follow up and the remaining needed surgical intervention. Among the patients infected with the rare species resolution of infection occurred in 3/7 (42.8%) patients and the difference was not significant ($P = 0.40$).

Discussion

Although statistically not significant, this study brings out the limitations of the Vitek 2 system in the identification of certain *Candida* species and non-*Candida* species when the reference identification was DNA sequencing of ITS1-5.8S-ITS2 regions of the rRNA gene. As such, the Vitek 2 yeast identification is a robust system with database that allows identification of 49 taxa of the most significant yeasts and yeast-like organisms. It is based on established biochemical methods and newly developed substrates.[26] An earlier study[27] has shown concordance of 91.4% between Vitek 2 and ITS sequencing in the identification of *Candida* species, however, we achieved a concordance of 56%. This difference may have been due to the fact that some isolates were unidentified and certain species were not present in the Vitek 2 database (*C. viswanathii, C. orthopsilosis*). We did find a significant correlation between Vitek 2 and sequencing when the confidence identification level of Vitek 2 was "excellent" and the identification "probability score" was more than 90%.

Notwithstanding the procedure of identification and contrary to earlier reports of *C. albicans* being the most common yeast in eye infections[28,29] this study confirms that *C. parapsilosis* is more commonly associated with eye infections compared with all other species. Most studies reporting yeast eye infections have found *C. parapsilosis* a close second to *C. albicans*. Our study records a reversal in the prevalence of these two species. It is possible that earlier studies using older methods were able to identify *C. albicans* by phenotypic methods, such as germ tube while other non-albicans species of *Candida* remained inadequately identified. Emergence of *C. parapsilosis* as an important neonatal blood stream and indwelling medical device pathogen has been reported.[30] Of the 21 isolates of *C. parapsilosis* in this study, 16 were from corneal scraping of patients with keratitis and 5 were from vitreous of patients with posttraumatic endophthalmitis.

As far as virulence factors are concerned our study showed that *C. albicans* remains more virulent than other species including *C. parapsilosis*. Significantly higher proportion of *C. albicans* isolates showed presence of virulence factors.

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TABLE 5. Clinical Details of Patients With Ocular Infections Associated With Rare Species of Yeast

Serial no.	Species	Clinical Diagnosis	Age, y/ Sex	Visual Acuity at Presentation, logMAR	Predisposing Factors	Topical Treatment	Systemic Treatment	Surgical/ Intravitreal Treatment	Duration of Follow Up, Days	Outcome	Best Corrected Visual Acuity at Last Follow Up
1	*Cyberlindnera fabianii*	Keratitis	22/M	2.7	Insect fall	Natamycin	Itraconazole	TPK	196	TPK	2.7
2	*Cyberlindnera fabianii*	Keratitis	60/M	2.7	nil	Natamycin	Ketoconazole	TABCL	10	Resolved	2.7
3	*Cyberlindnera fabianii*	Endophthalmitis	66/M	2.4	Cataract surgery with intraocular lens	Fluconazole	Ketoconazole	PPV + IOAB	14	Resolved	2.4
4	*Meyerozyma caribbica*	Keratitis	81/M	2.4	postPK (for Spheroidal degeneration) corneal infiltrate	Chlorocol	nil	TABCL	114	Resolved	1.8
5	*Meyerozyma caribbica*	Keratitis	45/F	2	nil	Natamycin	Keto	TPK	150	TPK	2.7
6	*Meyerozyma caribbica*	Keratitis	24/M	2.7	Injury with wood	Natamycin	nil	Evisc	61	Phthisis bulbi	3
7	*Meyerozyma caribbica*	Keratitis	60/M	2.7	Injury with wood	Natamycin	nil	Evisc	65	Eviseration	2.7

However, the proportion of isolates with biofilm forming potential was similar in both the species and majority of the isolates were susceptible to commonly used antifungals, such as amphotericin B and natamycin. In fact, with the exception of one *C. parapsilosis* all isolates in this study were susceptible to amphotericin B and natamycin.

The recommended treatment for *Candida* keratitis is amphotericin B or voriconazole.[31] In our earlier publication, we have described that of 12 patients with *Candida* keratitis who responded to medical therapy, eight were treated with natamycin and that natamycin may be administered for keratitis caused by *Candida* species. Over 88% (38/43) of isolates were susceptibile to caspofungin. A 5-year resistance surveillance report[32] in Brazilian hospital found 100% of *C. albicans* (blood stream infections) susceptible to fluconazole, in contrast to our results that found 42.8% of *C. albicans*, 47.6% of *C. parapsilosis*, and 66.6% of C. *viswanathii* to be resistant to fluconazole. Susceptibility to other drugs was variable with least susceptibility to itraconazole. Of all drugs tested, amphotericin B showed maximum in vitro efficacy for *Candida* spp. as well as non-*Candida* isolates, such as *Cyberlindnera fabianii* and *Meyerozyma caribbica* with comparable MIC_{50} for all three groups and no significant difference between MIC_{50} and MIC_{90} (Table 3). Although most of our patients were treated with antifungal drugs there were some who were either lost to follow up or needed surgical intervention such as evisceration and did not receive antifungal therapy (Tables 4, 5) resulting in poor outcome. There was no difference in the outcome of patients with *C parapsilosis* keratitis and patients infected with *Cyberlindnera fabianii* and *Meyerozyma caribbica*.

The susceptible isolates in this study were almost equally distributed among biofilm-positive and -negative isolates, thus establishing no relationship of biofilm forming potential to drug resistance among the isolates included in this study (Fig. 2f). The connection between biofilm and antibiotic resistance is complex. The quantitative correlation between biofilm formation and antibiotic resistance is currently unclear and there are conflicting reports in the literature. Studies on *Acinetobacter baumannii* have shown that population of bacteria with robust biofilm formation contain greater proportion of non-multidrug resistant isolates.[33] These authors have also shown that compared with susceptible isolates highly resistant isolates formed weaker biofilms but high-level of biofilm-specific resistance. The interesting explanation offered is that resistant isolates are not dependent on biofilm for their survival. This school of thought is strengthened by the observation that subminimum inhibitory concentration of certain antibiotics promotes biofilm formation suggesting stronger biofilm formation in the face of challenged susceptibility. Although the mechanism is not clear, it is known, at least in *Pseudomonas aeruginosa,* that expression of the β-lactamase gene ^{bla}TEM-1 inhibits biofilm formation by disrupting cell adhesion.[34] Some of these and our observations raise questions regarding the mechanisms of balance between biofilm formation and antibiotic resistance among microorganisms. Evaluation of a fairly large number of *Candida* spp. associated with eye infections in this study showed presence of a variety of virulence factors, however, we did not find any significant correlation of these factors with antifungal susceptibility. This study was not designed to correlate the virulence factors in pathogenic and nonpathogenic isolates of *Candida* as all our isolates were from patients with clinical disease. Further studies are required to look in to this aspect.

In conclusion, this series shows that *Candida parapsilosis* is the most common species of *Candida* associated with eye infections. The study describes the limitations of the Vitek 2

method in the identification of non-*Candida* spp. of yeast while there was no significant difference in the identification of *Candida* spp. by Vitek 2 and DNA sequencing methods. Continuous surveillance of serious infections caused by both albicans and non-albicans spp. of *Candida* is necessary for monitoring changes in the epidemiology. Among several drugs that were tested, *Candida* isolates showed susceptibility to amphotericin B and natamycin with lowest MIC_{50} of the former. Biofilm forming isolates showed marginally higher resistance to antibiotics such as caspofungin and fluconazole than nonbiofilm formers. Our study revealed that all *Candida* and non-*Candida* species had the maximum level of catalase, esterase activity, and some exhibited minimum level of phospholipase, proteinase, hemolytic activity, and no coagulase activity. Most of the species were positive for presence of *HWP1* over the *ASL1* gene, indicating the priority of *HWP1* gene in adhesion to tissues. Susceptibility to amphotericin B and natamycin remained unaffected by the various types of virulence factors exhibited by the isolates including biofilm formation.

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