Teo et al. BMC Infectious Diseases (2019) 19:63
https://doi.org/10.1186/s12879-019-3672-5

BMC Infectious Diseases

RESEARCH ARTICLE Open Access

Molecular mechanisms of azole resistance in *Candida* bloodstream isolates

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Abstract

Background: Antifungal resistance rates are increasing. We investigated the mechanisms of azole resistance of *Candida* spp. bloodstream isolates obtained from a surveillance study conducted between 2012 and 2015.

Methods: Twenty-six azole non-susceptible *Candida* spp. clinical isolates were investigated. Antifungal susceptibilities were determined using the Sensititre YeastOne® YO10 panel. The *ERG11* gene was amplified and sequenced to identify amino acid polymorphisms, while real-time PCR was utilised to investigate the expression levels of *ERG11*, *CDR1*, *CDR2* and *MDR1*.

Results: Azole cross-resistance was detected in all except two isolates. Amino acid substitutions (A114S, Y257H, E266D, and V488I) were observed in all four *C. albicans* tested. Of the 17 *C. tropicalis* isolates, eight (47%) had *ERG11* substitutions, of which concurrent observation of Y132F and S154F was the most common. A novel substitution (I166S) was detected in two of the five *C. glabrata* isolates. Expression levels of the various genes differed between the species but *CDR1* and *CDR2* overexpression appeared to be more prominent in *C. glabrata*.

Conclusions: There was interplay of various different mechanisms, including mechanisms which were not studied here, responsible for azole resistance in *Candida* spp in our study.

Keywords: Candida, Antifungal resistance, Genomics

Background

Candida bloodstream infections are an important healthcare issue known to be associated with high morbidity and mortality. There have been increasing reports of antifungal resistance. We have previously reported decreasing azole susceptibilities in our hospital, particular in *Candida tropicalis*. More than 20% of *C. tropicalis* were non-susceptible to fluconazole [1]. There are various mechanisms mediating azole resistance. It has been suggested that molecular mechanisms such as presence of mutations may be a predictive marker of clinical failure in *Candida* infections [2]. Whilst this has been established for echinocandin resistance, azole resistance mechanisms are not as well studied, particularly for non-albicans species. Elucidation of these mechanisms is crucial to make progress in understanding and treating invasive *Candida* infections.

Methods

In this study, we characterised the molecular mechanisms of azole resistance in 26 fluconazole non-susceptible *Candida* bloodstream isolates. These isolates were identified from a retrospective surveillance study conducted at a major regional tertiary referral hospital between 2012 and 2015 [1]. In brief, non-duplicate *Candida* bloodstream isolates from all adult inpatients (at least 21 years old) with temporally-related clinical signs and symptoms of infection admitted to the hospital during the study period were included. Antifungal susceptibility testing was performed using Sensititre YeastOne® YO10 panel (Trek Diagnostics System, West Sussex, England) according to manufacturer's recommendations. Minimum inhibitory concentrations were interpreted according to the current species-specific clinical breakpoints provided by the Clinical and Laboratory Standards Institute (CLSI)

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M27-S4 document or epidemiological cut-off values (ECV), where CLSI breakpoints were unavailable [3, 4]. For *Candida albicans* and *C. tropicalis*, isolates meeting the susceptible-dose-dependent (SDD) and resistant criteria were included, whereas only resistant *Candida glabrata* were included in this study. A total of 26 fluconazole non-susceptible isolates [*C. albicans* - 4/62 (6%); *C. glabrata* - 5/82 (6%); *C. tropicalis* - 17/78 (22%); *C. parapsilosis* - 0/35 (0%)] were identified from 257 *Candida* spp. isolates included in the surveillance study.

ERG11, *CDR1*, *CDR2* and *MDR1* gene expression were quantified in triplicates using real-time reverse transcription-PCR (RTPCR) with total RNA extracted from exponential-phase yeast peptone dextrose broth cultures on a CFX96 Real-Time PCR Detection System (Bio-Rad, USA). The primers used were adopted from previous publications [5–11], except for *C. glabrata CDR1* gene [F – TGGT GTTGCTAATGTCGCCA, R – GTCCCAAGTACTCG CCACAA] and *C. glabrata ERG11* gene [F – CCACCCATT GCACTCTTTGT, R – AGAACGTGGTAGTCCCTTGG]. Quantification of target genes was normalised to the level of *ACT1*, an endogenous reference gene. Relative gene expression was calculated as the fold change in expression of the isolates compared to the respective ATCC reference strains (*C. albicans* ATCC 90028, *C. glabrata* ATCC 2950, *C. tropicalis* ATCC 750). A fold increase of 3 times was considered to be an overexpression of the target gene. The *ERG11* gene was amplified and sequenced to identify amino acid mutations by comparing with reference wild-type GenBank sequences (*C. albicans* – X13296; *C. tropicalis* – M23673; *C. glabrata* – L40389).

Results

The susceptibility profiles of the isolates are displayed in Table 1. Cross-resistance to all azoles was observed in all isolates except for one *C. albicans* (CW138) and two *C.*

Table 1 Molecular characteristics of clinical fluconazole non-susceptible *Candida* spp. blood isolates

Isolate reference		MIC, ug/mL			Gene expression (fold increase)				Erg11p amino acid substitution(s)
		FLC	VRC	POS	*ERG11*	*CDR1*	*CDR2*	*MDR1*	
C. albicans	CW138	4 (SDD)	0.12 (S)	0.25 (NWT)	0.41	1.60	**9.01**	0.18	A114S, Y257H
	CW357	4 (SDD)	0.25 (SDD)	0.25 (NWT)	0.18	1.08	**5.79**	0.15	A114S, Y257H
	CW241	128 (R)	4 (R)	1 (NWT)	0.58	**4.49**	**122.50**	**141.28**	A114S, Y257H
	CW216	128 (R)	≥8 (R)	≥8 (NWT)	0.15	0.79	**3.32**	**4.86**	E266D, V488I
C. glabrata	CW193	64 (R)	4 (NWT)	≥8 (NWT)	0.46	**23.12**	**23.24**	N.A.	None
	CW262	64 (R)	0.25 (WT)	0.5 (WT)	1.08	**22.45**	**7.13**	N.A.	I166S
	CW378	64 (R)	4 (NWT)	2 (WT)	0.56	**4.02**	**8.08**	N.A.	None
	CW088	≥256 (R)	4 (NWT)	≥8 (NWT)	1.10	**19.78**	**14.55**	N.A.	None
	CW404	≥256 (R)	≥8 (NWT)	≥8 (NWT)	0.29	**18.70**	**6.92**	N.A.	I166S
C. tropicalis	CW190	4 (SDD)	0.25 (SDD)	0.5 (NWT)	0.39	**6.11**	N.A.	0.47	None
	CW219	4 (SDD)	0.5 (R)	0.5 (NWT)	0.95	1.56	N.A.	0.77	None
	CW361	4 (SDD)	0.5 (R)	0.25 (NWT)	2.38	0.27	N.A.	0.01	None
	CW395	4 (SDD)	0.5 (R)	0.5 (NWT)	0.45	0.63	N.A.	**3.27**	None
	CW071	8 (R)	0.25 (SDD)	0.5 (NWT)	0.11	1.28	N.A.	**30.53**	None
	CW018	16 (R)	0.25 (SDD)	0.25 (NWT)	0.61	2.75	N.A.	**23.42**	Y132F, S154F
	CW107	16 (R)	1 (R)	0.5 (NWT)	0.36	0.09	N.A.	**7.12**	Y132F, S154F
	CW178	16 (R)	0.5 (R)	1 (NWT)	2.83	**3.81**	N.A.	0.64	None
	CW385	16 (R)	0.5 (R)	1 (NWT)	0.12	**4.43**	N.A.	2.93	None
	CW263	64 (R)	4 (R)	0.12 (NWT)	**4.45**	1.95	N.A.	1.44	Y132F, S154F
	CW386	128 (R)	2 (R)	4 (NWT)	1.36	0.68	N.A.	1.33	None
	CW065	≥256 (R)	4 (R)	0.5 (NWT)	1.04	1.30	N.A.	**23.07**	Y132F, S154F
	CW067	≥256 (R)	≥8 (R)	1 (NWT)	0.11	0.93	N.A.	**7.04**	Y132F, S154F, F145 L
	CW192	≥256 (R)	≥8 (R)	1 (NWT)	1.36	0.76	N.A.	0.52	Y132F, S154F
	CW242	≥256 (R)	≥8 (R)	1 (NWT)	0.50	**6.10**	N.A.	1.21	Y132F, S154F
	CW266	≥256 (R)	4 (R)	0.25 (NWT)	0.27	**10.08**	N.A.	1.59	None
	CW271	≥256 (R)	≥8 (R)	1 (NWT)	**7.47**	1.30	N.A.	0.31	Y132F, S154F

FLC Fluconazole, *VRC* Voriconazole, *POS* Posaconazole, *S* Susceptible, *SDD* Susceptible dose-dependent, *R* Resistant, *WT* Wild-type, *NWT* Non wild-type, values in bold represent gene overexpression; underlined values represent heterozygous substitutions

glabrata (CW262 and CW378) isolates. All isolates retained susceptibility to other anti-fungals including anidulafungin, caspofungin, micafungin and amphotericin B (data not shown). In *C. albicans*, all isolates showed non-synonymous homozygous *ERG11* substitutions which included three distinct substitutions (A114S, Y257H and E266D). I166S substitutions were detected in two of the six *C. glabrata* isolates. Of the 17 *C. tropicalis* isolates, eight (47%) had *ERG11* substitutions. The most common substitutions were the concurrent observation of Y132F and S154F, which occurred primarily in resistant isolates with fluconazole MICs ≥8 μg/mL. Only two of the eight *ERG11* substitutions were homozygous, and there does not appear to be any correlation of the type of substitutions with MICs. The *ERG11* substitutions observed in all of the *Candida* spp. have been previously reported in literature except for I166S.

Among the different gene targets, it appeared that *ERG11* expression levels were mostly similar compared to the respective wild-type reference strains. *CDR2* expression was consistently elevated in fluconazole non-susceptible *C. albicans.* In the two resistant isolates with MIC 128 μg/mL, *MDR1* was also up-regulated. *CDR1* and *CDR2* co-expression was observed in all *C. glabrata* isolates. Gene overexpression was not consistent among *C. tropicalis* isolates – there were five isolates with *CDR1* overexpression and six isolates with *MDR1* overexpression. All *C. tropicalis* isolates only had overexpression of a single gene target. Interestingly, there were three *C. tropicalis* isolates with no *ERG11* mutations or any gene up-regulation.

Discussion

In this study, we evaluated the molecular mechanisms associated with azole resistance in various *Candida* species in our institution. Identification of antifungal susceptibilities through phenotypic methods such as MIC testing is often limited by the length of time required. Furthermore, current fungal MIC breakpoint interpretations are not supported by robust clinical data and are not predictive of clinical success/failure. Therefore, there is interest in identifying genotypic markers which could be rapidly identified for use in clinical prediction. Various previous studies have investigated different mechanisms of azole resistance in *Candida* species [5, 12–14]. Some of these studies have identified key *ERG11* substitutions which are associated with azole resistance e.g. Y132F, S154F [8, 15] and suggested that these mutations could be potential predictive markers of azole resistance.

In our context, it appeared that there was an interplay of various different mechanisms, including mechanisms which were not studied here, responsible for azole resistance in *Candida* spp. *ERG11* mutations were commonly detected in *C. albicans*, whereas the role of overexpression of azoles efflux pumps appeared to be more prominent in *C. albicans* (*CDR1*) and *C. glabrata* (*CDR1*, *CDR2*). In *C. tropicalis*, presence of Y132F and S154F substitutions was unable to explain the mechanisms of majority of our isolates. Less than half of the azole-resistant *C. tropicalis* harboured these amino acid substitutions. This was in contrast to the high frequency identified in another local study where > 90% of the isolates had Y132F and S154F substitutions [15]. Likewise, in another study, these mutations accounted for 95% of the fluconazole-resistant *C. tropicalis* [16].

Our study was limited by the small sample size although we had included all azole-resistant bloodstream isolates between 2012 and 2015. In addition, we did not perform further functional verification of the *ERG11* mutations and homology modelling experiments, therefore the clinical significance of I166S amino acid substitution in *C. glabrata* remains to be validated.

Conclusions

In conclusion, our results indicated that the mechanisms mediating azole resistance in our isolates are heterogeneous. There were isolates with unidentified resistance mechanisms warranting further exploration. Moving ahead, the use of more advanced molecular technologies such as next-generation sequencing might be considered for an in-depth molecular characterisation of azole-resistant *Candida* spp to aid the identification of potential resistance markers.

Abbreviations

ATCC: American Type Culture Collection; CLSI: Clinical and Laboratory Standards Institute; ECV: Epidemiological cut-off values; PCR: Polymerase chain reaction; RTPCR: Real-time reverse-transcription polymerase chain reaction

Acknowledgements

The authors acknowledge the excellent assistance of lab members of the Microbiology Lab, in particular Ms. Tan Mei Gie, and staff of the Pharmacy Research Lab, Singapore General Hospital, in the collection of the isolates. This study was presented in part at the 27th European Congress of Clinical Microbiology & Infectious Diseases, Vienna, Austria, 22nd – 25th April 2017 and the ASM Microbe 2017 meeting, New Orleans, USA, 1st – 5th June 2017.

Funding

This study was funded by grants from National Medical Research Council (NMRC/TA/0025/2013 and NMRC/CG/016/2013); and Pfizer Inc. (WS2347894). The study was also supported in part by Astellas Pharma Singapore Pte Ltd. with materials donation. The funders had no involvement in the study design, in the collection, analysis and interpretation of the data, or in the decision to submit the article for publication.

Availability of data and materials

Please contact corresponding author for data requests.

Authors' contributions

JQT, SJL, RSL, YC, TPL and ALT participated in the microbiological and/or molecular experiments. JQT and ALK conceived the study, interpreted the results, revised the manuscript and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The research protocol was approved by the Singhealth Centralised Institutional Review Board (2013/987/D). Informed consent was waived in view of retrospective nature of study.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 5 March 2018 Accepted: 2 January 2019
Published online: 17 January 2019

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