Rapid Molecular Detection of Echinocandin-Resistance in *Candida auris*

YC. Zhu[1], K. Hager[1], V. Chaturvedi[1, #], S. Chaturvedi[1, 2]

[1]Wadsworth Center Mycology Laboratory, New York State Department of Health, Albany, New York, USA
[2] Department of Biomedical Sciences, School of Public Health, University at Albany, Albany, New York, USA
[#] Current Address: Westchester Medical Center/New York Medical College, Valhalla, New York, USA

518-474-2892
yanchun.zhu@health.ny.gov

Abstract

Background: *Candida auris* is a multidrug-resistant yeast pathogen causing outbreaks in healthcare facilities worldwide, and the emergence of echinocandin-resistant *C. auris* is a concern. Currently used Clinical and Laboratory Standards Institute (CLSI) and commercial antifungal susceptibility tests are phenotype-based, slow, and not scalable, limiting their effectiveness in the surveillance of *C. auris*. There is an urgent need for accurate and rapid assessment of resistance to echinocandin-class antifungals, which are the first-line drugs of choice. We report developing a TaqMan chemistry probe-based fluorescence melt curve analysis (FMCA) following asymmetric PCR to assess mutations within the hot spot one (HS1) region of *FKS1,* encoding 1,3-β-D-glucan synthase, a target for echinocandins.

Methods: *C. auris* isolates exhibiting echinocandin resistance by broth microdilution method were obtained from our Culture Collection Repository. These were cultured, followed by DNA extraction, PCR amplification, and Sanger sequencing of *FKS1*. Multiple alignments of *FKS1* were done using Geneious 9.1.8 software. Primers and probes were designed from the HS1 region of the *FKS1* for FMCA on LightCycler® 480. Assay validation included crude DNA extraction in PBS-BSA of pure *C. auris* isolates and testing five microliters of extracted DNA in duplicate for assay reproducibility, limit of detection, specificity, blinded verification, and melting temperature (Tm) range.

Results: Twenty-five *C. auris* isolates were sequenced using ten primer pairs to produce full-length *FKS1* (~5600-bp). Multiple alignments revealed several mutations within the ~590-bp of *FKS1* comprising HS1 but not the HS2 region. Additional 39 isolates were sequenced for HS1 with three primer sets. Of 64 isolates, 25 were wild-type and 39 were mutants. Of mutants, one each was F635del and D642H/R645T, 4 were F635Y, 9 were F635C, 3 were S639P, 7 were S639F, and 14 were S639Y. All wild-type isolates were susceptible while all mutants were resistant to echinocandins except D642H/R645T by broth microdilution. FMCA correctly identified wildtype, F635C, F635Y, S639P, S639F, and D642H/R645T mutations with distinct Tm. An assay limitation was that it did not differentiate between S639F and S639Y as both yielded identical Tm's and F635del only produced a Tm against 639 but not against the 635 probe needing Sanger sequencing to confirm FMCA result. FMCA was highly specific and reproducible for identifying various mutations in *FKS1* for *C. auris* isolates, conferring echinocandin resistance. Additionally, there was excellent concordance between FMCA, Sanger sequencing, and antifungal susceptibility testing (AST).

Conclusions: Echinocandin-resistant *C. auris* pose a high risk for outbreaks worldwide. There is an urgent need for accurate and rapid assessment of echinocandin resistance as that is the drug of choice for patient management, infection control, and prevention programs. The FMCA assay developed in this study will aid in the shortcomings of the turnaround time faced with current phenotypic assays (AST) and sequencing used for resistant detection.

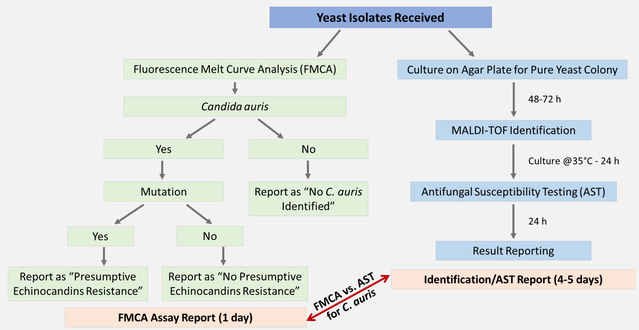
Introduction

Candida auris, a multidrug-resistant pathogenic yeast, has been reported in numerous countries on five continents, including Asia, Africa, Europe, South America, and North America, in the last decade (1-5). Antifungal resistance of *C. auris* is a major concern because variable resistance to all classes of antifungal drugs has been found, including resistance to echinocandins, which is the first-line drug of choice (6-10).

Accurate and rapid identification of echinocandin resistance is needed because CLSI and commercial antifungal susceptibility tests are phenotype-based, slow, and not scalable. Studies have found that echinocandin resistance is linked to mutations within the hot spot one (HS1) region of *FKS1*, encoding 1,3-β-D-glucan synthase (9-10). This study details the development of a Taqman chemistry probe-based fluorescence melt curve analysis (FMCA) following asymmetric PCR using the LightCycler® 480 platform to identify these mutations for a rapid molecular prediction of echinocandin resistance.

The flow chart below shows the testing algorithm upon implementing the FMCA assay to provide the most comprehensive results and a rapid turnaround time for appropriate infection control and patient care.

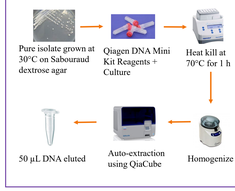
Clinical Yeast Isolate Testing



Methods

FKS1 Sanger Sequencing

DNA Extraction Flowchart



PCR/Sequencing

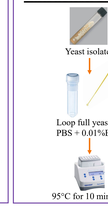
Conventional PCR cycle condition

Initial Denaturation		
95°C	3 min	1 cycle
PCR Amplification		
95°C	30 Sec	
40°C	30 sec	40 cycles
72°C	4 min	

Sequencing primers for full length *FKS1*

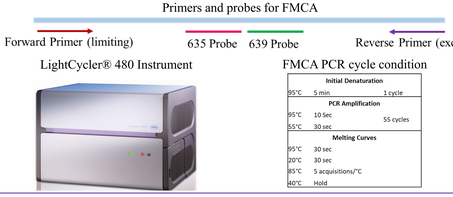
Fluorescence Melt Curve Analysis (FMCA) Assay

Crude DNA Extraction Flowchart



PCR Condition

Primers and probes for FMCA

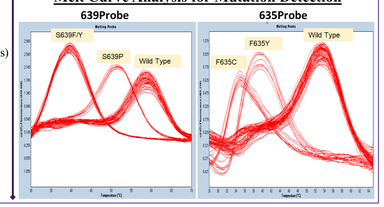


LightCycler® 480 Instrument

FMCA PCR cycle condition

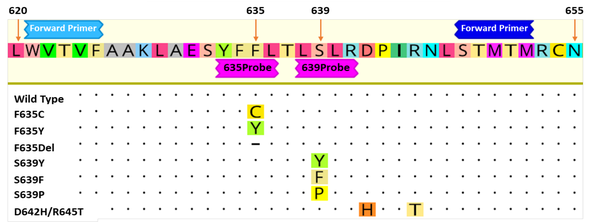
Initial Denaturation		
95°C	5 min	1 cycle
PCR Amplification		
95°C	10 Sec	55 cycles
55°C	30 sec	
Melting Curves		
95°C	30 sec	
20°C	30 sec	
85°C	5 acquisitions/°C	
40°C	Hold	

Melt Curve Analysis for Mutation Detection



Results

Multiple Alignments of HS1 region of *FKS1* & Design of Primers and Probes for FMCA



Amino Acid Key: F = Phenylalanine, C = Cysteine, Y = Tyrosine, P = Proline, H = Histidine, T = Threonine, S = Serine, D = Aspartic acid, R = Arginine

Concordance of Sanger Sequencing/FMCA/AST

No. of Isolates	FKS1 Sanger Sequencing Mutation	FMCA Assay			Echinocandin Resistance	Sanger vs. FMCA vs. AST Agreement
		635Probe Tm	639Probe Tm	Final Interpretation		
25	Wild Type	52.89-53.42	58.44-58.80	Wild Type	Susceptible	Yes
9	F635C	34.98-35.49	58.50-58.85	F635C	Resistant	Yes
4	F635Y	39.46-39.71	58.64-58.86	F635Y	Resistant	Yes
1	F635del	No Tm	58.64	Possible F635del	Resistant	Yes
14	S639Y	53.18-53.78	39.49-39.80	S639F/Y	Resistant	Yes
7	S639F	53.18-53.63	38.82-39.37	S639F/Y	Resistant	Yes
3	S639P	53.73-53.99	51.65-51.79	S639P	Resistant	Yes
1	D642H/R645T*	52.89	57.27	D642H/R645T	Susceptible	No

*D642H/R645T is a silent mutation, this mutation does not appear to implicate echinocandin resistance

FMCA Assay Validation

❖ **Melting Temperature (Tm) Range:** Tm range for each mutation was established by collection of data points for numerous *C. auris* wild-type and *FKS1* mutant isolates. Results showed unique Tm range for wild-type and several of the *FKS1* mutations.

635Probe Tm Range							
Mutation	**No. of Data points**	**Range**	**Mean**	**Median**	**Mode**	**SD**	**%CV**
F635C	113	34.24-35.68	35.02	35.03	34.92	0.25	0.72%
F635del	8	No Tm	No Tm	No Tm	No Tm	No Tm	No Tm
F635Y	73	37.83-40.11	39.35	39.47	39.71	0.50	1.27%
Wild Type	273	50.59-54.36	53.18	53.29	53.11	0.73	1.37%

639Probe Tm Range							
Mutation	**No. of Data points**	**Range**	**Mean**	**Median**	**Mode**	**SD**	**%CV**
S639F	59	37.08-39.81	38.77	38.78	38.88	0.54	1.39%
S639P	69	51.07-52.27	51.78	51.79	51.85	0.21	0.41%
D642H/R645T	53	56.54-57.89	57.35	57.43	57.43	0.32	0.56%
S639Y	155	38.8-40.13	39.67	39.74	39.73	0.28	0.70%
Wild Type	271	58.16-59.80	58.65	58.65	58.66	0.16	0.27%

❖ **Inter and Intra-assay Reproducibility:** The FMCA was reproducible with percent coefficient of variation below 5%.

Mutation	635Probe			639Probe		
	Mean Tm	SD	%CV	Mean Tm	SD	%CV
Wild Type	53.20	0.38	0.71%	58.57	0.18	0.30%
F635C	34.94	0.20	0.58%	58.47	0.13	0.23%
F635Y	39.14	0.63	1.60%	58.58	0.09	0.15%
S639F	53.59	0.88	1.64%	38.58	0.37	0.95%
S639P	53.55	1.09	2.03%	51.66	0.33	0.64%
D642H/R645T	53.74	0.47	0.87%	57.33	0.35	0.62%
S639Y	54.03	0.19	0.35%	39.19	0.22	0.57%

❖ **Sensitivity:** The limit of detection (LOD) was between 3 x 10^3 to 6 x 10^3 CFU/PCR reaction. The high LOD was not an issue as the assay was performed on pure *C. auris* isolates.

Mutation	CFU/PCR Reaction
Wild Type	6 x 10^3
F635C	3 x 10^3
F635Y	3 x 10^3
S639F	6 x 10^3
S639P	6 x 10^3
S639Y	6 x 10^3
D642H/R645T	6 x 10^3

❖ **Blinded Validation:** There was 100% concordance between FMCA and *FKS1* Sanger sequencing for all mutations except S639F/Y.

		FMCA							
		F635C	F635del	F635Y	S639F/Y	S639P	D642H/R645T	Wild Type	Negative
Sequencing	F635C	9	0	0	0	0	0	0	0
	F635del	0	1	0	0	0	0	0	0
	F635Y	0	0	4	0	0	0	0	0
	S639F	0	0	0	2* (16**)	0	0	0	0
	S639Y	0	0	0	14* (16**)	0	0	0	0
	S639P	0	0	0	0	3	0	0	0
	D642H/R645T	0	0	0	0	0	1	0	0
	Wild Type	0	0	0	0	0	0	25	0
	Negative	0	0	0	0	0	0	0	13

*Sequencing identified 2 as S639F and 14 as S639Y **FMCA identified 16 as S639F/Y

❖ **Specificity:** There was no cross-reactivity against various molds, and yeasts closely or distantly related to *C. auris* suggesting high specificity against *C. auris.*

	No. Isolates	Identified
***C. auris* (Clade I, II, III & IV)**	17	Yes
***Candida* spp. (Closely related to *C. auris*)**	7	No
***Candida* & Yeast spp. (Distantly related to *C. auris*)**	38	No
Molds	14	No

Conclusions

- We developed and validated a highly specific and reproducible FMCA assay for rapid identification of mutations in *FKS1* linked to echinocandin resistance in *C. auris*
- Benefits of FMCA assay:
  - Provides *C. auris* ID within 1 day vs. culture-based MALDI-TOF ID in 2-3 days
  - Provides presumptive echinocandin resistance in 1 day vs. culture-based AST in 4-5 days
  - Rapid turnaround time of *C. auris* ID and echinocandin resistance can assist epidemiologists and physicians in infection control and patient care
- Limitations of FMCA assay:
  - Unable to differentiate S639F and S639Y mutations due to identical Tm
  - Produced Tm only against 639 but not against 635 probe for F635del mutation, needing Sanger sequencing to confirm FMCA result

References

1. Lee WG, Shin JH, Uh Y, Kang MG, Kim SH, Park KH, et al. First three reported cases of nosocomial fungemia caused by *Candida auris*. *Journal of clinical microbiology*. 2011;49(9):3139-42.
2. Chowdhary A, Sharma C, Duggal S, Agarwal K, Prakash A, Singh PK, et al. New clonal strain of *Candida auris*, Delhi, India. *Emerging infectious diseases*. 2013;19(10):1670-3.
3. Wang X, Bing J, Zheng Q, Zhang F, Liu J, Yue H, et al. The first isolate of *Candida auris* in China: clinical and biological aspects. *Emerging microbes & infections*. 2018;7(1):93.
4. Magobo RE, Corcoran C, Seetharam S, and Govender NP. *Candida auris*-associated candidemia, South Africa. *Emerging infectious diseases*. 2014;20(7):1250-1.
5. Zhu Y, O'Brien B, Leach L, et al. Laboratory analysis of an outbreak of *Candida auris* in New York from 2016 to 2018—impact and lessons learned. *J Clin Microbiol* 2019. Epub December 18, 2019. 10.1128/JCM.01503-19
6. Lockhart SR, Etienne KA, Vallabhaneni S, Farooqi J, Chowdhary A, Govender NP, et al. Simultaneous Emergence of Multidrug-Resistant *Candida auris* on 3 Continents Confirmed by Whole-Genome Sequencing and Epidemiological Analyses. *Clinical infectious diseases: an official publication of the Infectious Diseases Society of America*. 2017;64(2):134-40.
7. Adams E, Quinn M, Tsay S, Poirot E, Chaturvedi S, Southwick K, et al. *Candida auris* in Healthcare Facilities, New York, USA, 2013-2017. *Emerging infectious diseases*. 2018;24(10):1816-24.
8. Ostrowsky B, Greenko J, Adams E, et al. Candida auris Isolates Resistant to Three Classes of Antifungal Medications - New York, 2019. *MMWR Morb Mortal Wkly Rep*. 2020;69(1):6-9. Published 2020 Jan 10. doi:10.15585/mmwr.mm6901a2
9. Kordalewska M, Lee A, Park S, et al. Understanding Echinocandin Resistance in the Emerging Pathogen Candida auris. *Antimicrob Agents Chemother*. 2018;62(6):e00238-18. Published 2018 May 25. doi:10.1128/AAC.00238-18
10. Sharma D, Paul RA, Rudramurthy SM, et al. Impact of *FKS1* Genotype on Echinocandin *In Vitro* Susceptibility in Candida auris and *In Vivo* Response in a Murine Model of Infection. *Antimicrob Agents Chemother*. 2022;66(1):e0165221. doi:10.1128/AAC.01652-21

Acknowledgements

This study was supported by the Clinical Laboratory Reference System, Wadsworth Center, NYSDOH, and Cooperative Agreement Grant (NU50CK000516), funded by the Centers for Disease Control and Prevention. We would also like to thank NYSDOH Epidemiology & Infection Control Programs, Wadsworth Center Media & Genomics Cores for help with various media for culture and sequencing of *C. auris*.