Video Article

Whole Genome Sequencing of *Candida glabrata* for Detection of Markers of Antifungal Drug Resistance

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Abstract

Candida glabrata can rapidly acquire mutations that result in drug resistance, especially to azoles and echinocandins. Identification of genetic mutations is essential, as resistance detected *in vitro* can often be correlated with clinical failure. We examined the feasibility of using whole genome sequencing (WGS) for genome-wide analysis of antifungal drug resistance in *C. glabrata*. The aim was torecognize enablers and barriers in the implementation WGS and measure its effectiveness. This paper outlines the key quality control checkpoints and essential components of WGS methodology to investigate genetic markers associated with reduced susceptibility to antifungal agents. It also estimates the accuracy of data analysis and turn-around-time of testing.

Phenotypic susceptibility of 12 clinical, and one ATCC strain of *C. glabrata* was determined through antifungal susceptibility testing. These included three isolate pairs, from three patients, that developed rise in drug minimum inhibitory concentrations. In two pairs, the second isolate of each pair developed resistance to echinocandins. The second isolate of the third pair developed resistance to 5-flucytosine. The remaining comprised of susceptible and azole resistant isolates. Single nucleotide polymorphisms (SNPs) in genes linked to echinocandin, azole and 5-flucytosine resistance were confirmed in resistant isolates through WGS using the next generation sequencing. Non-synonymous SNPs in antifungal resistance genes such as *FKS1*, *FKS2*, *CgPDR1, CgCDR1* and *FCY2* were identified. Overall, an average of 98% of the WGS reads of *C. glabrata* isolates mapped to the reference genome with about 75-fold read depth coverage. The turnaround time and cost were comparable to Sanger sequencing.

In conclusion, WGS of *C. glabrata* was feasible in revealing clinically significant gene mutations involved in resistance to different antifungal drug classes without the need for multiple PCR/DNA sequencing reactions. This represents a positive step towards establishing WGS capability in the clinical laboratory for simultaneous detection of antifungal resistance conferring substitutions.

Video Link

The video component of this article can be found at https://www.jove.com/video/56714/

Introduction

Candida glabrata is an increasingly encountered pathogen with importance as a species that exhibits resistance to the azoles as well as more recently, to the echinocandins[1,2,3]. Unlike the diploid *C. albicans*, the haploid genome of *C. glabrata* may allow it to acquire mutations and develop multi-drug resistance more easily. Co-resistance to both drug classes has also been reported[4]. Hence, early evaluation of antifungal susceptibility and detection of drug resistance in *C. glabrata* is crucial for correct, targeted therapy as well as in the context of antifungal stewardship to limit drivers of antimicrobial resistance[1,5,6]. Establishing an efficient workflow to rapidly detect the presence of confirmatory mutations linked to resistance biomarkers in resistant isolates will also help to improve prescribing decisions and clinical outcomes.

Antifungal susceptibility is usually assessed by measuring minimum inhibitory concentration (MIC) which is defined as the lowest drug concentration that results in a significant reduction in growth of a microorganism compared with that of a drug-free growth control. The Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) have standardized susceptibility testing methods in order to make MIC determination more accurate and consistent[7,8]. However, the utility of antifungal MIC remains limited especially for the echinocandins, in particular with regards to inter-laboratory comparisons where varying methodologies and conditions are used[9]. There is also uncertain correlation of MICs with response to echinocandin treatment and inability to distinguish WT (or susceptible) isolates from those harboring FKS mutations (echinocandin-resistant strains)[10,11]. Despite the availability of confirmatory single-gene PCRs and Sanger sequencing of antifungal resistance markers, realization of results is often delayed due to lack of simultaneous detection of multiple resistance markers[5,12]. Hence, concurrent detection of resistance-conferring mutations in different locations in the genome, enabled by whole genome sequencing-based analysis, offers significant advantages over current approaches.

Whole genome sequencing (WGS) has been successfully implemented to track disease transmission during outbreaks as well as an approach for genome-wide risk assessment and drug resistance testing in bacteria and viruses[13]. Recent advances in nucleic acid sequencing technology have made the whole genome sequencing (WGS) of pathogens in a clinically actionable turn-around-time both technically and economically feasible. DNA sequencing offers important advantages over other methods of pathogen identification and characterization employed in microbiology laboratories[14,15,16]. First, it provides a universal solution with high throughput, speed and quality. Sequencing can be applied to any of microorganisms and allows economies of scale at local or regional laboratories. Second, it produces data in a 'future-proof' format amenable to comparison at national and international levels. Finally, the potential utility of WGS in medicine has been augmented by the rapid growth of public data bases containing reference genomes, which can be linked to equivalent data bases that contain additional clinical and epidemiological metadata[17,18].

Recent studies have demonstrated the utility of WGS for identification of antifungal resistance markers from clinical isolates of *Candida spp*.[10,19,20]. This is mostly due to the availability of high-throughput benchtop sequencers, established bioinformatics pipelines and decreasing cost of sequencing[21,22]. The advantage of fungal WGS over Sanger sequencing is that WGS allows sequencing of multiple genomes on a single run. In addition, WGS of *Candida* genomes can identify novel mutations in drug targets, track genetic evolution, and emergence of clinically relevant sequence-types[20,22,23]. Most importantly, in cases of intrinsic multidrug resistance, WGS can assist in early detection of resistance-conferring mutations prior to treatment selection[22,24].

Here, we examined the feasibility of WGS-enabled screening for mutations associated with drug resistance to different classes of antifungal agents. We present a methodology for the implementation of WGS from end-user and diagnostic mycology laboratory perspectives. We included in this analysis three isolate pairs cultured from three separate clinical cases in which *in vitro* resistance to the echinocandins and 5-flucytosine developed over time following antifungal treatment.

Protocol

No ethical approval was required for this study.

1. Subculture and inoculum preparation for *Candida glabrata*

1. Select a panel of *C.glabrata* isolates to be studied which should also include at least one *C. glabrata* American Type Culture Collection (ATCC) with known susceptibility pattern.
2. Subculture an isolate by touching a single colony using a sterile disposable plastic loop and streaking onto a Sabouraud's dextrose agar (SDA) plate[8].
3. Incubate the SDA plate for 24-48 h at 35 °C for pure culture of isolate with good growth.

2. Determination of Antifungal Susceptibility

1. Use a sterile disposable plastic loop to pick 4-5 colonies of approximately 1 mm diameter from a freshly subcultured *C. glabrata* isolate on SDA plate. Resuspend in 3 mL of sterile distilled water. Mix well by gentle pipetting to obtain uniform suspension.
2. Adjust the cell density to 0.5 McFarland which is equivalent to 1 x 10^6 to 5 x 10^6 cells/mL using a densitometer [8].
3. Perform susceptibility testing using commercial assay (see Table of Materials and Reagents) on all *C. glabrata* isolates following manufacturer's instructions. Interpret the susceptibility of isolates based on resultant MICs of antifungal drugs according to CLSI guidelines and prepare report (Table 1)[8].

3. Genomic DNA extraction for sequencing

1. Resuspend a loopful of colonies from a freshly grown SDA plate in 300 µL of 50 mM EDTA in a 1.5 mL tube.
2. Add 40 µL of zymolyase (10 mg/mL) to the suspension, and gently pipet 5 times until suspension is uniform.
3. Incubate the sample at 37 °C for 1-2 h to digest the cell wall. Cool at room temperature for 5 min.
4. Centrifuge the suspension at 14,000 × *g* for 2 min and then carefully remove the supernatant.
5. Extract genomic DNA following DNA extraction kit guidelines (see Table of Materials).
6. Resuspend extracted DNA pellet in 50 µL of 10 mM Tris Buffer (pH 7.5-8.5) instead of the elution buffer provided in the kit.
7. Check the purity of DNA by measurement of optical density (O.D) at 260/280 nm[25].

4. Genomic DNA quantification

1. Prepare a 1x Tris EDTA (TE) buffer provided in the assay kit based on manufacturer's guidelines for the fluorescence assay (see Table of Materials).
2. Dilute the DNA sample by adding 2 µL to 98 µL of 1x TE assay buffer (final volume of 100 µL, dilution factor 1:50) in a disposable 96 well plate. This dilution step can be performed either manually using a multichannel pipette or by automated liquid handling workstation.
3. Prepare the range of standards by diluting the lambda DNA (100 µg/mL) provided in the fluorescence assay kit (Table 2) and include for measurement along with samples.
4. Add 100 µL of the fluorescent dye to 100 µL diluted DNA samples and standards for the reaction. Incubate for 5 min at room temperature, protected from light.
5. Measure the fluorescence of all samples based on manufacturer's guidelines.
6. Plot the standard curve using the fluorescence readings and calculate the original concentration of the DNA samples.
7. Determine the volume of DNA and 10 mM Tris buffer (pH 8) to be added to adjust the DNA concentration to 0.2 ng/µL.
8. Perform the DNA dilutions using automated liquid handling workstation. This step can be also achieved by manual pipetting.

5. DNA Library Preparation

Note: Library preparation and sequencing was performed following manufacturer's protocols and guidelines provided by company (**Figure 1A**) (see Table of Materials).

1. **Tagmentation and PCR amplification**
    1. Label a new 96-well hard-shell thin wall plate.
    2. Add 5 µL of quantified input DNA at 0.2 ng/µL (1 ng in total) to each sample well of the plate.
    3. Add 10 µL of tagmentation buffer and 5 µL of amplification buffer to each well containing DNA and gently pipette to mix. Seal the plate with an adhesive plate seal.
    4. Place the plate in a thermal cycler and run the following PCR program: 55 °C for 5 min, and hold at 10 °C. When the sample reaches 10 °C, proceed immediately to neutralize.
    5. Add 5 µL of neutralization buffer to the plate to neutralize the amplicon reaction, and gently pipette mix. Seal the plate and incubate at room temperature for 5 min.
    6. Add 15 µL of indexing PCR mastermix to the samples.
    7. Use a box of index primer tubes available for a setup of 96-well plate format so that each sample gets a unique combination of indices based on index template (Table 3).
    8. Arrange the index primer tubes in the index plate rack (**Figure 1B**) using the order provided in the template on Table 3 and record the position of the indices on the template.
    9. Place the tagmentation plate with added PCR mastermix on the index plate rack with the index tubes in order.
    10. Put index primer 1 tubes in vertical arrangement, and index primer 2 tubes in horizontal arrangement on the index rack. Using a multichannel pipette, carefully add 5 µL of index primers to each sample.
    11. Replace old caps of index tubes with new caps to avoid cross-contamination between indices.
    12. Seal the plate using 96-well clear plate sealers and perform the second PCR as follows: 95 °C for 30 s, 12 cycles of 95 °C for 10 s, 55 °C for 30 s, 72 °C for 30 s and 72 °C for 5 min.

2. **PCR Cleanup**
    1. Transfer the PCR product for PCR-cleanup, from the tagmentation plate to a deep-well plate (see Table of Materials).
    2. Vortex the commercial magnetic beads solution (see Table of Materials) based on manufacturer's instructions and add 30 µL of beads to each PCR product in deep-well plate.
    3. Shake the plate on a microplate shaker at 1800 rpm for 2 min and incubate at room temperature without shaking for 5 min.
    4. Place the plate on a magnetic stand for 2 min until the supernatant has cleared.
    5. Discard the supernatant carefully with the plate still on the magnetic stand.
    6. Add 200 µL of freshly prepared 80% ethanol with the plate on the magnetic stand.
    7. Incubate the plate on the magnetic stand for 30 s and carefully remove and discard the supernatant without disturbing the beads.
    8. Repeat the wash step again and allow the beads to air-dry for 15 min. Remove excess ethanol if any.
    9. Remove the plate from the magnetic stand and add 52.5 µL of resuspension buffer to the beads.
    10. Shake the plate on a microplate shaker at 1800 rpm for 2 min and incubate at room temperature for 2 min without shaking.
    11. Place the plate on the magnetic stand and allow the supernatant to clear.
    12. Using a multichannel pipette, carefully transfer 50 µL of the supernatant from the cleanup plate to a new hard-shell plate.

3. **Library Normalization**
    1. Thaw library normalization reagents according to manufacturer's guidelines (see Table of Materials).
    2. Transfer 20 µL of the supernatant from the cleanup plate to a new deep-well plate.
    3. Add 45 µL of magnetic bead suspension and seal the plate with a plate sealer.
    4. Shake the plate on a microplate shaker at 1800 rpm for 30 min. This incubation time is critical and should not be exceeded.
    5. Place the plate on a magnetic stand for 2 min and confirm that the supernatant has cleared (**Figure 1B**).
    6. Remove and discard the supernatant in an appropriate hazardous waste container with the plate still on the magnetic stand.
    7. Remove the plate from the magnetic stand and wash the beads with 45 µL wash buffer.
    8. Shake the plate with wash buffer on a microplate shaker at 1800 rpm for 5 min.
    9. Place plate on magnetic stand for 2 min and discard supernatant when it turns clear.
    10. Remove the plate from the magnetic stand and repeat the wash with wash buffer again.
    11. Remove the plate from the magnetic stand and add 30 µL of 0.1 N NaOH.

12. Shake the plate with 0.1 N NaOH on a microplate shaker at 1800 rpm for 5 min and place the plate on the magnetic stand for 2 min or until the liquid is clear.
13. Add 30 µL of elution buffer to each well of a new 96-well hard-shell thin wall final normalized library plate.
14. Transfer 30 µL of supernatant from normalization plate to final normalized library plate to make final volume 60 µL. The libraries are now ready to be sequenced.

4. **Determination of DNA Library Concentration by qPCR**
    1. Thaw the mastermix, primers and standards provided in the qPCR kit according to manufacturer's guidelines (see Table of Materials).
    2. Combine the PCR reagent mastermix and primer provided in the qPCR kit following manufacturer's instructions and aliquots can be stored at -20 °C.
    3. To determine the DNA library concentration, make a 1/8000 dilution of the DNA libraries using 10 mM Tris buffer (pH 8) by performing a 1:100 dilution (1.5 µL DNA library to 148.5 µL Tris buffer) followed by a 1:80 dilution (2 µL from 1:100 to 158 µL Tris buffer).
    4. Shake the dilution plate at 700 rpm for at least 1 min and then centrifuge at 14000 × *g* for 1 min.
    5. Prepare 20 µL of final PCR reaction by mixing 4 µL of diluted DNA library or DNA standards and 16 µL of mastermix.
    6. Perform PCR following manufacturer's settings in thermocycler: 95 °C for 5 min, 35 cycles of 95 °C for 30 s and 60 °C for 45 s, and final 65 °C to 95 °C for melt curve analysis.
    7. Obtain the Ct values of the sample DNA libraries and standards from the qPCR thermocycler.
    8. Generate a standard curve from the Ct value of the standards (**Figure 2**). Define the upper and lower QC range by ± 3 cycles from the mean. For example, if the mean Ct value is 13 cycles then the QC range is between 16 and 10 cycles.
    9. Determine the individual and average library concentrations (ALC) from the standard curve and Ct values.
    10. Determine volume of total pooled libraries (PAL) to be used based on calculation given below for final sequencing considering that target library concentration is between 1.4-1.8 pM.
        Note: Average library concentration obtained from qPCR= ALC; Total Pooled Libraries (PAL) = ALC / 2; Denatured PAL (DAL) = PAL * 0.666
        Depending on the volume of DAL that is mixed with buffer, is the concentration of library to be added to the flow cell. For example, if 65 µL of library is added to 835 µL of buffer, then from this dilution (Dil 1) 195 is added to a total volume of 1300 µL: (65/900) * dnPAL = Dil1
        Dil1 * (195/1300) = Final Concentration (should be between 1.4-1.8 pM)

6. Library pooling and Initiating Sequencing in Benchtop Sequencer

1. Thaw reagent cartridge according to manufacturer's guidelines. Take out a new flow cell from its package from 4 °C storage and bring to room temperature atleast 30 min prior to sequencing. Take out buffer cartridge and prechill sequencing buffer before use (see Table of Materials).
2. Prepare a control library by mixing 5 µL of library (1 nM) and 5 µL of 0.2 N NaOH. Vortex briefly and incubate for 5 min at room temperature to denature the control library into single strands.
3. Add 5 µL of 200 mM Tris-HCl, pH 7 and vortex. Add 235 µL prechilled sequencing buffer and mix gently. The total volume is 250 µL with control library final concentration at 20 pM.
4. Pool DNA libraries by transferring 5 µL of each sample library to be sequenced from the final normalized library plate into a single low-bind 1.5 mL tube.
5. Add 30 µL of pooled library and 30 µL of 0.2 N NaOH to denature libraries in another low bind tube.
6. Vortex the low-bind tube and incubate for 5 min at room temperature to denature libraries into single strands.
7. Add 30 µL of 200 mM Tris-HCl, pH 7 to tube with denatured libraries to neutralize reaction.
8. Add 65 µL of neutralized denatured libraries suspension and 835 µL of pre-chilled sequencing buffer and vortex to mix well.
9. In a final low bind tube combine the following: 195 µL from neutralized denatured libraries, 1.30 µL of control library and 1103.70 µL of sequencing buffer. Mix properly.
10. Load the final library mix (1300 µL) into the designated spot on reagent cartridge.
11. Set-up the sequencing run by entering project and sample details in the Sequencer designated website following guidelines.
12. Initiate sequencing following guidelines. Load flow cell, reagent cartridge with libraries and buffer cartridge in benchtop sequencer.
13. Record batch numbers of all reagent kits and cartridges used in the sequencing.

7. Data Download from Sequencing Website

1. Download FASTQ files following manufacturer's instructions provided on website.
2. For a good quality run check that the percentage Q30 is ≥ 75% and cluster density is between 170-280 K/mm^2 with optimal at 200-210 K/mm^2 (Table 4).

8. Sequencing Data analysis

1. Import FASTQ files of sequenced samples into data analysis integrated software package (see Table of Materials).
2. Create a sequencing workflow in software by adding features from list namely trimming, mapping to reference (select reference genome), local realignment and variant analysis (**Figure 3A**) using settings listed in Table 5.
3. Run workflow by selecting a single sample or a batch of sample FASTQ files and save output files in designated sample folders.
4. Generate report for sequence coverage depth, mapped regions and list of structural variants in genome (**Figure 3B**).
5. Use list of structural variants to search for non-synonymous single nucleotide polymorphisms (SNPs) in genes conferring resistance and virulence.

6. Prepare report by listing SNP location, gene, and number of resistant or susceptible isolates (Table 6).

Representative Results

Thirteen *C. glabrata* comprising *C. glabrata* ATCC 90030 and 12 isolates from the Clinical Mycology Reference laboratory (isolates CMRL1 to CMRL12), Westmead Hospital, Sydney were studied (Table 1). These included three pairs of isolates CMRL-1/CMRL-2, CMRL-3/CMRL-4 and CMRL-5/CMRL-6 obtained before and after antifungal therapy with no epidemiological links between them [24] (Table 1).

The MICs were determined using CLSI interpretative breakpoint for nine antifungal agents namely, Amphotericin (AMB), Anidulafungin (ANI), Micafungin (MIF), Caspofungin (CAS), 5-Flucytosine (5-FC), Posaconazole (POS), Voriconazole (VRC), Itraconazole (ITR) and Fluconazole (FLC). *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality control strains. Among the isolate pair, CMRL-1 and CMRL-2, the second isolate CMRL-2 was resistant to caspofungin (MIC 8 vs. 0.12 mg/L, Table 1). CMRL-2 also had similar proportional increases in the MICs of anidulafungin and micafungin (≥ 0.5 mg/L) resulting in *in vitro* resistance to all three echinocandins[24]. Likewise, isolate CMRL-4 had developed echinocandin resistance than those of isolate CMRL-3 (Table 1). Between the third pair, isolate CMRL-6 had a 5-flucytosine MIC (>64 vs. 0.06 mg/L)[24]. Both these isolate pairs were susceptible/wild-type (WT) to other antifungal agents tested. Isolate CMRL-6 and CMRL-12 were found to be resistant or non-WT to all azoles. CMRL-7 was resistant to fluconazole and non-WT to voriconazole (Table 1). *C. glabrata* ATCC 90030 and isolates CMRL-8 to CMRL-11 were susceptible susceptible/WT to all antifungal agents[24].

WGS of 13 isolates was performed using the benchtop sequencer. On an average, a mid-output sequencing run, yielded around 27-40 GB data with an error rate between 0.5-1.4%. The average percentage Q30 obtained was usually around 80-85%. The flow-cell cluster density (K/mm^2) ranged between 200-250 (Table 4). The raw sequence data from this study have been deposited at NCBI Sequence Read Archive (SRA) under the project number PRJNA310057. 98% of sequencing reads mapped to the *C. glabrata* reference genome (strain CBS138) through analysis in integrated data analysis software. The average read depth coverage was 75x with average read length of 143-bp. Structural variant detection identified more than 50, 000 SNPs per isolate. Particularly, when analyzing the strain pairs CMRL1/CMRL-2, total SNPs found on each isolate were around 79,000, for CMRL-3/CMRL-4, the total number of SNPs were around 60,000 and for CMRL-5/CMRL-6, more than 56,000 when compared to CBS138[24]. The SNP difference when compared between isolates in a strain pair was less than 25.

Based on the susceptibility profile of the isolates, known resistance biomarkers were selected for analysis[24], particularly, *FKS1*, *FKS2* and *FKS3* (echinocandin resistance), *FCY1* and *FCY2* (5-flucytosine resistance), and *ERG9, ERG11, CgCDR1, CgPDR1* and *CgFLR1* (azole resistance). The genes were checked for known mutations, and frequency of SNP occurrences. Only non-synonymous SNPs in genes with read depth coverage of ≥20 i.e., high-quality SNPs (hq-SNPs) were specifically studied.

Notably, *FKS* mutations were identified in the genome of both echinocandin-resistant isolates[24]. Of the first two pairs, the echinocandin resistant isolates, CMRL-2 harbored a single *FKS2* mutation S629P, and CMRL-4, the *FKS2* mutation S663P (Table 6). Of the third pair, a SNP in *FCY2* (Ala237Thr) was found in both CMRL-5 (5-flucytosine susceptible) and CMRL-6 (resistant) (Table 6). However, SNPs in *FCY2* were also found in other phenotypically-WT isolates (CMRL-1, CMRL-2, CMRL-10)[24]. Isolates CMRL-6 and CMRL-12 were noteworthy for their pan-azole resistant/non-WT character and had SNPs in both *CgCDR1* (encoding azole efflux pumps) and *CgPDR1* (encoding the transcription factor regulating the efflux pumps) (Table 6)[26,27]. The presence of mutations in another efflux pump gene, *CgFLR1* occurred in both azole-susceptible, and azole-resistant isolates[26,28]. Investigation for SNPs within the *ERG9* (coding squalene synthase) revealed mutations but there were no mutations identified in *ERG11*[24].

WGS analysis also revealed multiple non-synonymous SNPs in *Candida* cell wall adhesion genes namely, *EPA1, EPA6, PWP2* and *PWP5*. *EPA6* mutations were present in 9/12 isolates. SNPs in *PWP2* and *PWP5* were also present in almost all isolates, except isolates CMRL-1 and CMRL-11[24].

Isolate	AMB	ANI	MIF	CAS	5-FC	POS	VRC	ITR	FLC	Interpretation of antifungal susceptibility	Genes Conferring Resistance Identified by WGS
CMRL-1	0.5	0.03	< 0.008	0.12	< 0.06	0.5	0.12	0.25	8	Susceptible to all	-
CMRL-2	0.5	1	1	8	< 0.06	0.25	0.06	0.12	4	Resistant to all echinocandins only	FKS1
CMRL-3	0.25	0.015	0.015	0.12	< 0.06	1	0.25	0.5	8	Susceptible to all	-
CMRL-4	2	1	1	> 8	< 0.06	0.5	0.12	0.25	8	Resistant to all echinocandins only	FKS2
CMRL-5	1	0.12	0.015	0.12	< 0.06	1	0.5	0.5	16	Susceptible to all	-
CMRL-6	1	0.06	0.015	0.06	> 64	> 8	8	> 16	256	Resistant to 5-FC and azoles	FCY2, CgPDR1, CgCDR1
CMRL-7	0.25	0.06	0.015	0.25	< 0.06	1	8	0.5	256	Resistant to all azoles only	CgPDR1, CgCDR1, CgFLR1
CMRL-8	0.5	0.03	0.008	0.06	< 0.06	0.5	0.25	0.25	4	Susceptible to all	-
CMRL-9	1	0.03	0.015	0.25	< 0.06	1	0.5	1	16	Susceptible to all	-
CMRL-10	1	0.03	< 0.008	0.5	< 0.06	1	0.5	0.5	16	Susceptible to all	-
CMRL-11	0.5	0.03	< 0.008	0.03	< 0.06	0.5	0.25	0.5	8	Susceptible to all	-
CMRL-12	0.5	0.03	0.015	0.06	< 0.06	> 8	2	8	128	Resistant to all azoles only	CgPDR1, CgCDR1, CgFLR1
ATCC 90030	1	0.03	0.015	0.06	< 0.06	1	0.5	0.5	8	Susceptible to all	-

Abbreviations: MIC, minimum inhibitory concentration; AMB, amphotericin B; ANI, anidulafungin; CAS, caspofungin; FLC, fluconazole; ITR, itraconazole; MIF, micafungin; POS, posaconazole; VRC, voriconazole; 5-FC, 5-flucytosine.

Table 1. *In vitro* susceptibility of 13 *Candida glabrata* isolates including CMRL-1/CMRL-2, CMRL-3/CMRL-4 and CMRL-5/CMRL-6 isolate pairs obtained before and after antifungal therapy

Standards	DNA Standard Volume (µL)	1X TE buffer	Reagent (µL)	Total in 96-well plate (µL)	Final DNA Concentration (ng/mL)
Std-Pico 1	8 (Standard DNA tube 100 µg/mL)	1992	100	200	1000
Std-Pico 2	10 (from Std-Pico 1)	90	100	200	100
Std- Pico 3	5 (from Std-Pico 1)	95	100	200	50
Std-Pico 4	2 (from Std-Pico 1)	198	100	200	10
Std-Pico 5	10 (Std-Pico 4)	90	100	200	1
Blank	-	100	100	200	Blank

Table 2. Protocol for Preparing Standards for Standard Curve Generation for DNA Quantification

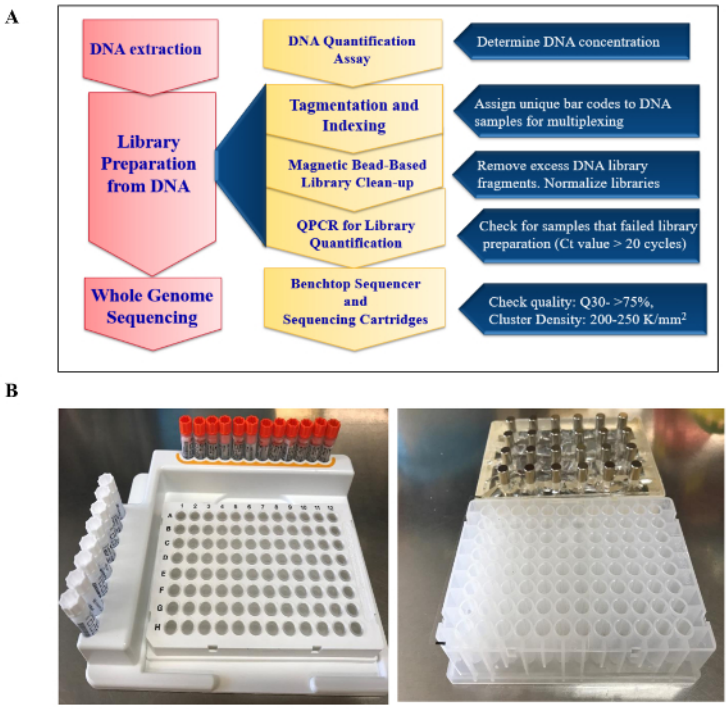

Figure 1. Sequencing Workflow: (A) An outline of major analytical steps for library preparation and sequencing on a benchtop sequencer. **(B)** Important components for DNA library preparation such as the (left to right) index plate rack for arrangement of indices during indexing and magnetic rack with deep 96-well plate for magnetic bead based clean-up of DNA libraries. Please click here to view a larger version of this figure.

		1	2	3	4	5	6	7	8	9	10	11	12
	Index Set A	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
A	S502	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
B	S503	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24
C	S505	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30	Sample 31	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36
D	S506	Sample 37	Sample 38	Sample 39	Sample 40	Sample 41	Sample 42	Sample 43	Sample 44	Sample 45	Sample 46	Sample 47	Sample 48
E	S507	Sample 49	Sample 50	Sample 51	Sample 52	Sample 53	Sample 54	Sample 55	Sample 56	Sample 57	Sample 58	Sample 59	Sample 60
F	S508	Sample 61	Sample 62	Sample 63	Sample 64	Sample 65	Sample 66	Sample 67	Sample 68	Sample 69	Sample 70	Sample 71	Sample 72
G	S510	Sample 73	Sample 74	Sample 75	Sample 76	Sample 77	Sample 78	Sample 79	Sample 80	Sample 81	Sample 82	Sample 83	Sample 84
H	S511	Sample 85	Sample 86	Sample 87	Sample 88	Sample 89	Sample 90	Sample 91	Sample 92	Sample 93	Sample 94	Sample 95	Sample 96
		1	2	3	4	5	6	7	8	9	10	11	12
	Index Set B	N716	N718	N719	N720	N721	N722	N723	N724	N726	N727	N728	N729
A	S502	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
B	S503	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24
C	S505	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30	Sample 31	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36
D	S506	Sample 37	Sample 38	Sample 39	Sample 40	Sample 41	Sample 42	Sample 43	Sample 44	Sample 45	Sample 46	Sample 47	Sample 48
E	S507	Sample 49	Sample 50	Sample 51	Sample 52	Sample 53	Sample 54	Sample 55	Sample 56	Sample 57	Sample 58	Sample 59	Sample 60
F	S508	Sample 61	Sample 62	Sample 63	Sample 64	Sample 65	Sample 66	Sample 67	Sample 68	Sample 69	Sample 70	Sample 71	Sample 72
G	S510	Sample 73	Sample 74	Sample 75	Sample 76	Sample 77	Sample 78	Sample 79	Sample 80	Sample 81	Sample 82	Sample 83	Sample 84
H	S511	Sample 85	Sample 86	Sample 87	Sample 88	Sample 89	Sample 90	Sample 91	Sample 92	Sample 93	Sample 94	Sample 95	Sample 96
		1	2	3	4	5	6	7	8	9	10	11	12
	Index Set C	N701	N702	N703	N704	N705	N706	N707	N710	N711	N712	N714	N715
A	S513	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
B	S515	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24
C	S516	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30	Sample 31	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36
D	S517	Sample 37	Sample 38	Sample 39	Sample 40	Sample 41	Sample 42	Sample 43	Sample 44	Sample 45	Sample 46	Sample 47	Sample 48
E	S518	Sample 49	Sample 50	Sample 51	Sample 52	Sample 53	Sample 54	Sample 55	Sample 56	Sample 57	Sample 58	Sample 59	Sample 60
F	S520	Sample 61	Sample 62	Sample 63	Sample 64	Sample 65	Sample 66	Sample 67	Sample 68	Sample 69	Sample 70	Sample 71	Sample 72

G	S521	Sample 73	Sample 74	Sample 75	Sample 76	Sample 77	Sample 78	Sample 79	Sample 80	Sample 81	Sample 82	Sample 83	Sample 84
H	S522	Sample 85	Sample 86	Sample 87	Sample 88	Sample 89	Sample 90	Sample 91	Sample 92	Sample 93	Sample 94	Sample 95	Sample 96
		1	2	3	4	5	6	7	8	9	10	11	12
	Index Set D	N716	N718	N719	N720	N721	N722	N723	N724	N726	N727	N728	N729
A	S513	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
B	S515	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24
C	S516	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30	Sample 31	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36
D	S517	Sample 37	Sample 38	Sample 39	Sample 40	Sample 41	Sample 42	Sample 43	Sample 44	Sample 45	Sample 46	Sample 47	Sample 48
E	S518	Sample 49	Sample 50	Sample 51	Sample 52	Sample 53	Sample 54	Sample 55	Sample 56	Sample 57	Sample 58	Sample 59	Sample 60
F	S520	Sample 61	Sample 62	Sample 63	Sample 64	Sample 65	Sample 66	Sample 67	Sample 68	Sample 69	Sample 70	Sample 71	Sample 72
G	S521	Sample 73	Sample 74	Sample 75	Sample 76	Sample 77	Sample 78	Sample 79	Sample 80	Sample 81	Sample 82	Sample 83	Sample 84
H	S522	Sample 85	Sample 86	Sample 87	Sample 88	Sample 89	Sample 90	Sample 91	Sample 92	Sample 93	Sample 94	Sample 95	Sample 96

Table 3. Template of Different Index Arrangement

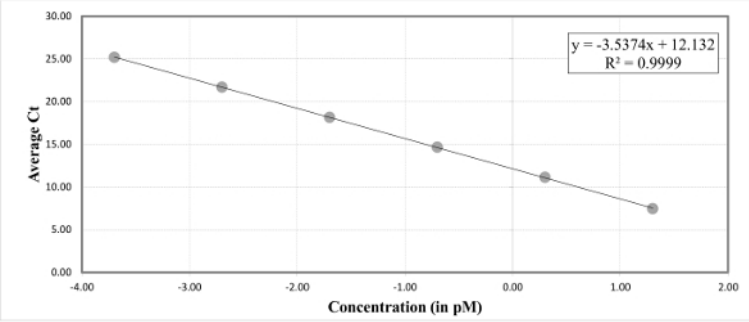

Figure 2: A standard graph generated with the Ct values of standards. Use the standard curve intercept and slope values to determine the average library concentration from Ct value of sample libraries in duplicates. Please click here to view a larger version of this figure.

Metrics	**Standard Values**	**Values Obtained (Average)**
Yield	32.5-50 Gb for mid-output	42 Gb for mid-output
	100-130 Gb for high-output	120 Gb for high-output
Percentage Q30	≥ 75%	80%
Cluster Density	170-230 K/mm^2, Optimal at 200 K/mm^2	210 K/mm^2.
Clusters Passing Filters	> 70%	89.09%
Intensity by Cycle	Above 1000 for each tile in flowcell	Above 1500 for each tile in flowcell
Error Rate	Below 1.5	1.4

Table 4. Metrics for a standard sequencing run in Benchtop Sequencer

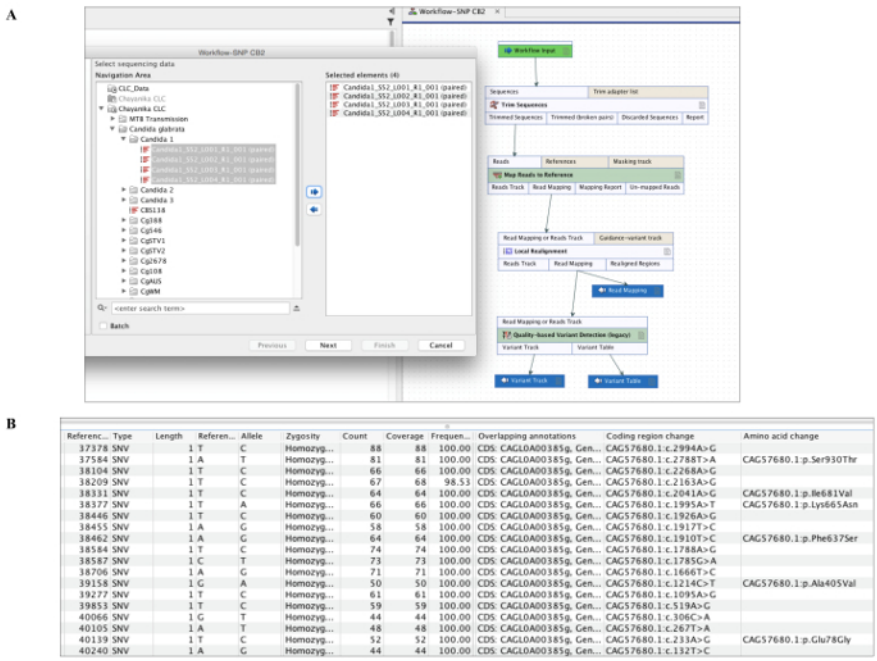

Figure 3: Sequencing Data Analysis Software. (A) Create a desired workflow including sequence trimming, mapping to reference and quality based variant detection. Run workflow by selecting sample FASTQ files (individual or multiple samples in batch). **(B)** List of structural variants showing reference position, coverage, nucleotide change and amino acid change obtained for analysis of sample sequences. Please click here to view a larger version of this figure.

1. Trim sequences	**Setting Used**
Trim Bases	Default settings
Filter on length with maximum number of nucleotide in reads	1000
Filter on length with minimum number of nucleotide in reads	50
2. Mapping Reads to reference	
Reference selected	CBS138
Reference masking with masking mode	No masking
Mapping Options	Default settings
3. Local Realignment	
Alignment settings	Default settings
4. Quality Based Variant Detection	
Neighbourhood radius	5
Minimum gap and mismatch count	2
Minimum neighbourhood quality	15
Minimum central quality	20
Read filters	Default settings
Minimum coverage	4
Minimum variant frequency (%)	75
Variant filters	Default settings
Maximum expected alleles (Ploidy)	2
Genetic code	Standard

Table 5. Workflow Parameters and Settings on the Software

Structural Variant Nucleotide Position	Gene	Drug(s)	Found in Number of Isolates	Resistant Isolates	Susceptible Isolates
SNP_Cg_95352_S629P_fks1	CgFKS1	CAS, ANI, MIF	1	CMRL-2	-
SNP_Cg_375361_S633P_fk2	CgFKS2	CAS, ANI, MIF	1	CMRL-4	-
SNP_Cg_285870_A237T_fcy2	CgFCY2	5-FC	2	CMRL-6	CMRL-5
SNP_Cg_286311_I384F_fcy2	CgFCY2	5-FC	2	0	CMRL-1, CMRL-2
SNP_Cg_720995_C128F_erg9	CgERG9	FLC, POS, VRC, ITR	3	CMRL-6	CMRL-5, CMRL-10
SNP_Cg_48683_R376Q_pdr1	CgPDR1	FLC, POS, VRC, ITR	1	CMRL-6	-
SNP_Cg_50384_G250N_pdr1	CgPDR1	FLC, POS, VRC, ITR	1	CMRL-7	-
SNP_Cg_49640_P695L_pdr1	CgPDR1	FLC, POS, VRC, ITR	1	0	CMRL-11
SNP_Cg_49858_N768D_pdr1	CgPDR1	FLC, POS, VRC, ITR	1	CMRL-12	-
SNP_Cg_203787_H58Y_cdr1	CgCDR1	FLC, POS, VRC, ITR	5	CMRL-6, CMRL-12	CMRL-5, CMRL-10, CMRL-11
SNP_Cg_205529_M638I_cdr1	CgCDR1	FLC, POS, VRC, ITR	1	CMRL-7	-
SNP_Cg_206938_N1108S_cdr1	CgCDR1	FLC, POS, VRC, ITR	1	CMRL-7	-
SNP_Cg_589884_I116V_flr1	CgFLR1	FLC, POS, VRC, ITR	4	CMRL-7	CMRL-1, CMRL-2, CMRL-9
SNP_Cg_589470_V254I_flr1	CgFLR1	FLC, POS, VRC, ITR	1	CMRL-12	-

Table 6. Report of structural variant position in genes linked to antifungal drug resistance found in the number of isolates of *C. glabrata*

Discussion

This study determined feasibility, approximate timelines and precision of WGS-guided detection of drug resistance in *C. glabrata*. The turnaround time (TAT) for the library preparation and sequencing was four days and reporting of analyzed results one-two days. This compares with at least a similar amount TAT for susceptibility assays from culture plates and Sanger sequencing with significantly higher number of samples. Around 30-90 *C. glabrata* genomes can be sequenced based on sequencing flow-cell capacity, with 80-100% sequencing coverage. Since the sequencing was performed on an in-house WGS laboratory setup, the costs/resource requirements in this study was equivalent to current costs of Sanger sequencing and probe-based assays with an estimated cost of AUD 80-100 per sample. Susceptibility of all isolates were determined by a commercial assay kit that were read visually using a reading mirror. Culture growth was indicated by change in colorimetric growth indicator from blue to pink. MIC was read as the first blue well after a series of pink (growth) wells i.e., the lowest concentration of the antifungal agent that substantially inhibits growth. For WGS, genomic DNA libraries were prepared using the library preparation kit. The isolate libraries were quantified by qPCR. The quantified libraries were pooled together for the final sequencing run performed in the benchtop sequencer.

In our analysis, the presence of SNPs in genes conferring resistance in isolates correlated well with the elevated *in vitro* MIC against the drugs. The mutations*S629P in FKS1* and S663P in *FKS2* identified were clearly associated with resistance to echinocandins. Both mutations are well-known to confer phenotypic resistance[29,30]. Simultaneous genome-wide sequencing also revealed mutations in genes linked to 5-flucytosine (gene *FCY2*) and azole resistance (*CgPDR1*, *CgCDR1* and *CgFLR1*) that are associated with resistance through activation/overexpression as efflux pumps[26,27,28]. However, the effects of mutations in genes linked to azole and 5-flucytosine resistance[31] needs to be confirmed by additional functional analyzes to assess the gene expression level. Interestingly, large number of SNPs were also found in cell wall adhesins in all isolates[32,33]. Mutations in adhesion gene *EPA6,* which encodes biofilm formation, occurred relatively frequently[33]. Additionally, isolates CMRL-3, CMRL-4 and CMRL-8 that lacked mutations in azole resistance genes had no documented SNPs in *EPA1* and *EPA6*[24,34]. However, overall, no specific association between the SNPs in adhesins and drug MICs could be found due to the low number of test isolates included.

The implementation of WGS in clinical mycology requires the implementation of robust quality management system. High quality genomic DNA and quality control checkpoints that accompany each step in the experiment is essential for a good WGS outcome. The purity of *C. glabrata* sample DNA submitted for library preparation can be validated using UV absorbance method (absorbance at 260/280 nm and 260/230 nm[25]. In our experience, for *C. glabrata* DNA the 260/280 is expected to be within the recommended range of 1.8-2 and for 260/230 between 2-2.2. If DNA extracts do not meet these quality requirements, additional purification by ethanol precipitation can be performed. Tagmentation is an essential step of adding unique bar code sequences called index primers so as enable differentiation of multiple sample during sequencing (multiplexing). Hence, in the indexing process, extra caution must be taken to avoid cross-contamination between index tubes and samples

in order to maintain uniqueness of the indices. Normalization in sequencing ensures that any differences arising in the sample DNA libraries that might introduce bias in sequencing data is eliminated. A normalization step using a bead based clean-up usually allows removal of excess DNA library fragments and variations in library size that may arise during library preparation. Therefore the 30 min incubation is critical for optimal cluster density. Cluster density which is the density of the clonal clusters of sample libraries generated by massive amplification during sequencing influences data quality like Q30 scores and total data output. While underclustering might give high data quality, it will also result in lower data output whereas overclustering low Q30 scores. The DNA libraries should be free of magnetic bead residue during PCR cleanup and normalization as that can interfere with sequencing data quality. QPCR should be performed on at least a few representative sample libraries including a reference strain as a positive control (the ATCC strain of *C. glabrata* in this case) from a particular set of indices. The average Ct values should be between 15 - 18 cycles. Any sample within this range is considered acceptable for the sequencing run. However, if the Ct values are outside this range the qPCR should be repeated. Samples can sometimes fail qPCR either due to low quality input DNA or error during library preparation. Based on calculations from the positive control, the minimum concentration of the libraries that will produce good sequence data should be established. Using the average concentration of the libraries obtained from qPCR, the volume of final library to be added for sequencing can be calculated. This should produce the recommended final library concentration between 1.4-1.8 pM. For *C. glabrata* libraries, the established Ct range using the *C. glabrata* ATCC isolate was between 16-18 cycles with an average library concentration range of 300-500 pM. The corresponding volume of pooled denatured DNA libraries was within the range of 60-65 µL for a cluster density range between 200-250 K/mm^2.

The sequence data files should be demultiplexed before further analysis. This can be achieved by sorting into their respective libraries using their bar codes after sequencing has taken place. An optional step of quality trimming of the FASTQ files can be performed prior to data analysis. The sequence reads of the isolates from WGS were analyzed using a custom workflow created in a standard software package. This allowed trimming of low-quality reads and then mapping to the reference *Candida* genome. The reference genome, CBS138, was downloaded from *Candida* Genome Database (CGD) and linked to the workflow prior to analysis. The presence of DNA repeats in the *Candida* genome (for example, adhesin genes have DNA repeats) may complicate the alignment of short-read sequences and the SNP calling. This can be improved by the additional local realignment of mapped sequences. The output from the workflow was a structural variants list that provided annotated genes with non-synonymous and synonymous SNP positions and coverage. This was used to identify SNPs in genes of interest and prepare the final analysis report for the isolates (Table 6).

Some limitations of our study and approach should be acknowledged. Our report is based on small number of isolates and there is no standard fungal resistome database for clinical mycology. Although Sanger DNA sequencing is currently more accessible to clinical laboratories, it has limited ability to simultaneously detect multiple gene mutations that confer drug resistance in *Candida* spp. (>20 *FKS* mutations for echinocandin resistance alone). With consolidation of pathology services and decreasing sequencing costs, the practicality of using WGS over Sanger sequencing, is likely to increase. However, an important consideration is the initial laboratory setup and cost of WGS instrument implementation as well as end-user training of laboratory personnel. Long term costs would essentially include, procuring sequencing reagent kits, and accessories. Additional cost and a higher TAT should be expected if the WGS instrument is not in-house. Furthermore, the presence of DNA repeat sequences in genomes may complicate the alignment of short-read sequences and the SNP calling. The availability of high-quality reference genomes sequenced using long-read technologies will help to sustain the quality of SNP identification.

In future, WGS is expected to bring improvements in clinical therapy by fast-tracking reporting time in diagnostic settings that is easily accessible and interpreted by clinicians[20,22]. This can be achieved with development of curated and up-to-date WGS antifungal drug resistance databases of novel and confirmatory mutations to infer antifungal resistance. As more genomes of clinically relevant yeasts of known phenotypic drug susceptibility become available for analysis, novel markers and mechanisms of antifungal drug resistance are likely to be discovered. These developments will substantially reduce risks of treatment failures due to multi drug resistance and drug toxicity. In conclusion, the next generation sequencing with appropriate quality management protocols enables genome-wide detection of mutations conferring resistance in *Candida glabrata* which can augment phenotypic testing in mycology laboratories. The insights provided by rapid genome sequencing of clinically relevant *Candida spp* can fundamentally change our understanding of mechanisms of resistance to different classes of antifungal agents and improve the management of patients with invasive fungal diseases.

Disclosures

The authors have no competing financial interests and no conflict of interest to disclose.

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