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Karius Cell-Free DNA Metagenomic Assay of Plasma Detects Pulmonary and Disseminated *Trichosporon* Infections in Patients With Hematological Malignancies

Tempe K. Chen[1], Asim A. Ahmed[2], Kathryn Goggin[3], Josh Wolf[3], Julie Chu[4], William Pomputius[4], Benjamin Briggs[5], Melissa Sanacore[6], Mehlhem Solh[6], Kent Holland[6], Asad Bashey[6], Lawrence Morris[6], Scott Solomon[6], Samuel Webster[6], and Thomas J. Walsh[7]

[1]Miller Children's and Women's Hospital Long Beach, University of California Irvine, Long Beach, CA [2]Karius, Inc., Redwood City, CA [3]St. Jude Children's Research Hospital, Memphis, TN
[4]Children's Minnesota, Minneapolis/ St. Paul, MN [5]University of California San Francisco, San Francisco, CA [6]Northside Hospital Cancer Institute, Atlanta, GA
[7]Weill Cornell Medicine of Cornell University, New York Presbyterian Hospital, New York, NY, USA

Correspondence:
Thomas J. Walsh, MD, FIDSA
Weill Cornell Medicine of Cornell University
1300 York Avenue, Room A-421
New York, NY 10065
Telephone: (212) 746-9968
Email: thw2003@med.cornell.edu

Abstract

Trichosporon species are uncommon but emerging pathogens that cause life-threatening infections that are resistant to amphotericin B and echinocandins. Diagnosis of deeply invasive trichosporonosis is often elusive to conventional culture methods until locally advanced or disseminated disease has advanced. We found that next-generation sequencing of microbial cell-free DNA from plasma using the Karius-based technology established a diagnosis in six pediatric and adult patients. The median age was 17 yrs (8-72 yrs). All patients had underlying acute leukemia. Six patients had pulmonary nodules and two also had multiple cutaneous lesions with negative blood cultures. Biopsies of one pulmonary nodule and of two cutaneous lesions revealed yeast-like cells. Culture of cutaneous lesions in two patients grew *Trichosporon* sp. Karius assays performed on plasma and analyzed against a database of more than 300 species of fungi identified *Trichosporon asahii* in two cases, *Trichosporon* spp. in three, *Trichosporon faecale* in one. In all cases, the metagenomic results defined therapy for treatment of invasive trichosporonosis, which is based in antifungal triazoles. In one patient with pulmonary nodules, lung biopsy revealed mixed septate and non-septate hyphal elements that later grew *Trichosporon asahii*, *Rhizomucor pusillus*, and *Candida parapsilosis*, all of which were identified by Karius assay. In summary, invasive *Trichosporon* spp. are uncommon polyene and triazole-resistant pathogens that cause life-threatening invasive fungal disease in immunocompromised patients with hematological malignancies; next-generation sequencing of fungal cell-free DNA by Karius-based technology identified all six cases of pulmonary and disseminated trichosporonosis, contributing to early detection and pathogen-directed antifungal therapy.

Introduction

Trichosporon species are re-emerging pathogens in immunocompromised patients that are resistant to echinocandins and to the fungicidal activity of amphotericin B. These organisms cause life-threatening pulmonary and disseminated infections in neutropenic hosts with hematological malignancies. With the expanding use of echinocandins for antifungal prophylaxis and treatment, *Trichosporon* spp. are re-emerging to cause serious infections. Early diagnosis and initiation of appropriate therapy with an antifungal triazole is essential for survival and a favorable outcome.

We report herein the use of cell-free rapid molecular diagnosis using next-generation sequencing of microbial cell-free DNA to identify disseminated infections and refractory pulmonary infiltrates caused by these resistant fungal pathogens. The cell-free next-generation sequencing technology was critical in identifying *Trichosporon* spp. in half of the patients when all other modalities had failed to yield the diagnosis.

Materials and Methods

The methods described in this study are those developed and implemented by Karius. (https://www.karuisdx.com/).

Sample Collection and Processing
Blood samples (5 mL) were collected in BD vacutainer plasma preparation tubes. Within 1 hour of sample collection, tubes were spun down at 1,100 RCF for 10 min at room temperature. Samples were shipped overnight to Karius, Inc. (Redwood City, CA).

Measurement of cfDNA using NGS
Cell-free DNA was extracted from plasma, NGS libraries were prepared, and sequencing was performed on an Illumina NextSeq®500. Sequencing reads identified as human were removed, and remaining sequences were aligned to a curated pathogen database. Any of over 1,000 organisms in the Karius clinical reportable range found to be present above a predefined statistical threshold were reported as previously described (Hong et al., 2018). The quantity for each organism identified was expressed in Molecules Per Microliter (MPM), the number of DNA sequencing reads from the reported organism present per microliter of plasma. For additional detailed methods, see Supplemental Appendix.

Reference Database and QC
Reference genomes for Homo sapiens and microorganisms (bacteria, viruses, fungi/molds, and other eukaryotic pathogens) were retrieved from the National Center for Biotechnology Information (NCBI) ftp site (NCBI, U.S. National Library of Medicine (NLM), Human Genome: https://www.ncbi.nlm.nih.gov/genome/guide/human/, release GRCh38.p7), (NCBI, U.S. NLM, Microbial Genomes: https://www.ncbi.nlm.nih.gov/genome/microbes/). Sequence similarities between microorganism references were inspected to identify taxonomic mislabeling and sequence contamination. From the reference genomes passing these quality controls, a subset was selected to maximize sequence diversity. As part of the selection process, NCBI BioSample data (NCBI, U.S. NLM, BioSample: https://www.ncbi.nlm.nih.gov/biosample/), were used to ensure the inclusion of reference genomes from both clinical and non-clinical isolates. The final reference genome dataset included over 21,000 reference genomes, containing over 2.7 million sequences. Selected sequences were collected into a single FASTA file and used to generate our microorganism reference BLAST database. A subset of these taxa, including 1251 clinically significant microorganisms, was used as the clinical reportable range.

Clinical Reportable Range (CRR)
The selection of organisms in our clinical reportable range (CRR) was performed as follows. A candidate list was generated by two board-certified Infectious Disease physicians by including (a) DNA viruses, culturable bacteria, additional fastidious and unculturable bacteria, mycobacteria, and eukaryotic pathogens from the standard text [1] and a number of infectious disease references, (b) organisms in the pathogen database referenced in published case reports and (c) reference genomes sequenced from human clinical isolates (as indicated by NCBI's BioSample resource) with publications supporting pathogenicity. Organisms from the above list that were associated with high quality reference genomes, as determined by our reference database QC process (see above), were used to further narrow the range. Finally, organisms at risk of generating common false-positive calls because of sporadic environmental contamination were removed. The sequence database is continuously curated to minimize human cross-reactivity as well as cross-reactivity between pathogens and is screened to mitigate contamination with sequences from human or other organisms.

Sequencing
Plasma samples were thawed, centrifuged at 16,000 rcf for ten minutes, and spiked with a known concentration of synthetic DNA molecules for quality control purposes. Cell-free DNA was extracted from 0.5 mL plasma using a magnetic bead-based method (Omega Biotek, Norcross, GA). DNA libraries for sequencing are constructed using a modified Ovation® Ultralow System V2 library preparation kit (NuGEN, San Carlos, CA). Negative controls (buffer only instead of plasma) and positive controls (healthy plasma spiked with a known mixture of microbial DNA fragments) were processed alongside patient samples in every batch. Samples were multiplexed with other samples and sequenced on an Illumina NextSeq® 500.

Analysis Pipeline
Primary sequencing output files were processed using bcl2fastq (v2.17.1.14) to generate the demultiplexed sequencing reads files. Reads were filtered based on sequencing quality and trimmed based on partial or full adapter sequence. The bowtie2 (version 2.2.4) tool was used to align the remaining reads against Karius' human and synthetic-molecules references. Sequencing reads exhibiting strong alignment against the human references or the synthetic molecule references were collected and excluded from further analysis. Remaining reads were aligned against Karius' proprietary microorganism reference database using NCBI-blast (version 2.2.30+). A mixture model was used to assign a likelihood to the complete collection of sequencing reads that included the read sequence probabilities and the (unknown) abundances of each taxon in the sample. An expectation-maximization algorithm was applied to compute the maximum likelihood estimate of each taxon abundance. Only taxa whose abundances rejected the null hypothesis of originating from environmental contamination (as calculated from the negative controls) at high significance levels were reported. The quantity for each organism identified was expressed in Molecules Per Microliter (MPM), the number of DNA sequencing reads from the reported organism present per microliter of plasma. The entire process from DNA extraction through analysis was typically completed within 28 hours.

Results

We found that next-generation sequencing of microbial cell-free DNA from plasma using the Karius-based technology established a diagnosis in six pediatric and adult patients.

- Median age was 17 yrs (8-72 yrs)
- All patients had underlying acute leukemia
- 6 patients had pulmonary nodules; two had multiple cutaneous lesions
- One pulmonary nodule and two cutaneous lesions revealed yeast-like cells
- Culture of cutaneous lesions in two patients grew *Trichosporon* sp.
- Karius assays performed on plasma identified *Trichosporon asahii* in five cases and *Trichosporon faecale* in one
- In all cases, the metagenomic results defined therapy for treatment of invasive trichosporonosis, which was based in antifungal triazoles

Table 1: Clinical and Diagnostic Characteristics

Case	Age/ Sex	Diagnosis	Clinical Manifestations	Karius assay (mpm)	Histology/ Microbiology
1	14F	AML	fever, neutropenia, pulmonary nodules	*Trichosporon faecale* (38)	positive histology, culture negative
2	65M	AML	fever, LUL infiltrate and L pleural effusion	*Trichosporon asahii* (1,008) *Rhizomucor pusillus* (2,433) *Candida parapsilosis* (216)	positive histology, culture negative
3	72M	CMML --> AML	fever, hemoptysis, pneumonia, rash	*Trichosporon asahii* (47)	BAL negative
4	8M	AML	paronychia, pulmonary nodules, renal lesion, disseminated subcutaneous nodules	*Trichosporon asahii* (1,031) *Escherichia coli* (848) *Staph haemolyticus* (108)	culture positive
5	8M	ALL	fever, neutropenia, maculopapular rash on face and body, chorioretinitis, splenic and renal microabscesses, septic shock	*Trichosporon asahii* (111,732) *Lactobacillus rhammnosus* (219)	culture positive
6	19F	AML	fever, widespread papular rash, pulmonary nodules	*Trichosporon asahii* (3,976)	positive histology, culture positive

Table 2: Management and Outcome

Case	Antifungal Prophylaxis	Antifungal Therapy	Outcome
1	micafungin	micafungin isavuconazole posaconazole	survival
2	micafungin	micafungin posaconazole liposomal amphotericin B	death
3	N/A	micafungin isavuconazole posaconazole voriconazole	survival
4	posaconazole isavuconazole	liposomal amphotericin B caspofungin isavuconazole voriconazole	death
5	micafungin	voriconazole liposomal amphotericin B terbinafine	survival
6	posaconazole	liposomal amphotericin B voriconazole	survival

Illustrative Case

A 14 year-old female following induction chemotherapy for AML presented with fever, persistent neutropenia, and nodular lung lesions diagnosed by Karius' cell-free DNA metagenomic assay of plasma as *Trichosporon faecale* pneumonia. Patient developed this infection while receiving micafungin for antifungal prophylaxis during neutropenia.

Routine surveillance PET/CT obtained after induction chemotherapy was consistent with nodular pneumonia of fungal etiology (Figure 1). She underwent thoracoscopic wedge resection of the lung nodule and Karius assay was ordered prior to the procedure. Histopathology revealed nodular necrotizing granulomas of the lung. GMS stain demonstrated fungal forms consisting of hyphal elements, budding yeasts, and arthroconidia which were thought to be morphologically compatible with *Trichosporon* spp. (Figure 2). While intraoperative cultures ultimately were negative, the Karius assay was positive for *Trichosporon faecale* DNA.

Patient was initially empirically treated with liposomal amphotericin B. Once histopathology and Karius assay results returned, treatment with isavuconazole was initiated and continued for 1 year. In follow-up over two years, the patient's pulmonary trichosporonosis resolved and her leukemia remains in full remission.

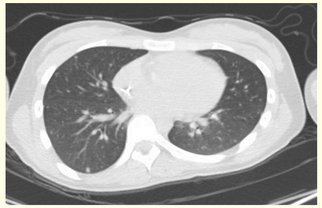

Figure 1: CT scan demonstrating nodular pulmonary infiltrates proven to be caused by *Trichosporon faecale*

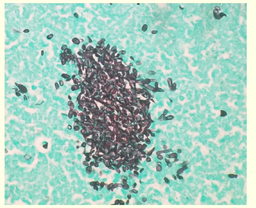

Figure 2: GMS stain demonstrating hyphal elements, budding yeasts, and arthroconidia histologically compatible with *Trichosporon* spp.

Conclusion

Given the importance of targeted antifungal triazole therapy for treatment of serious diseases caused by *Trichosporon* spp., the Karius cell-free DNA metagenomic assay of plasma ushers in a new modality of individualized medicine that may greatly advance the diagnosis and management of pulmonary and disseminated trichosporonosis.