RESEARCH ARTICLE

Next-generation sequencing of microbial cell-free DNA for rapid noninvasive diagnosis of infectious diseases in immunocompromised hosts [version 1; peer review: awaiting peer review]

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Abstract

Background: Cell-free DNA (cfDNA) sequencing has emerged as an effective laboratory method for rapid and noninvasive diagnosis in prenatal screening testing, organ transplant rejection screening, and oncology liquid biopsies.

Methods: Here we report our experience using next-generation sequencing (NGS) for detection of microbial cfDNA in a cohort of ten immunocompromised patients with febrile neutropenia or deep-seated infection.

Results: Among five hematological malignancy patients, for whom a microbiological diagnosis was established, pathogen identification by cfDNA NGS demonstrated 100% positive agreement with conventional diagnostic laboratory methods. Further, cfDNA identified the etiological agent in two patients with culture negative sepsis who had undergone hematopoietic stem cell transplant.

Conclusion: These data support the clinical utility of measurement of microbial cfDNA sequencing from peripheral blood for rapid noninvasive diagnosis of infections in immunocompromised hosts. Larger studies are needed.

Keywords

Cell-free microbial DNA, next generation sequencing, infection, immunocompromised host, hematopoietic stem cell transplant
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Competing interests: Karius, Inc. ran the tests on the clinical specimens for these 10 patients at no charge to our institution.

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Introduction
Infections are a leading cause of morbidity and mortality among immunocompromised individuals\textsuperscript{1–4}. Bacteremia occurs in up to 25% of all patients with neutropenia and fever\textsuperscript{5}. Infection is a leading cause of non-relapse mortality among hematopoietic cell transplantation (HCT) recipients\textsuperscript{6}. The incidence of bacteremia\textsuperscript{7–9} and double-stranded DNA viral reactivation\textsuperscript{10} is higher than 40% and 90%, respectively, within the first 100 days post-transplant. The cumulative incidence rates of proven/probable invasive fungal infections during the first year after allogeneic HCT with non-myeloablative conditioning is 19%\textsuperscript{11}. Infection is also a common complication of chimeric antigen receptor-modified T (CAR-T)-cell immunotherapy with 28-day cumulative incidence of 23% after CAR-T-cell infusion\textsuperscript{12}.

Establishing a microbiological diagnosis of infectious diseases in this vulnerable population is often challenging for a number of reasons. i) Prior exposure to antibiotics and anti-fungals which confounds the yield of blood cultures; indeed, most patients with neutropenia and fever will have no infectious etiology documented\textsuperscript{1}. ii) Low sensitivity of mycobacterial and fungal cultures; some microorganisms, such as fastidious bacteria, mycobacteria and dimorphic fungi require longer incubation periods; and blood cultures in almost half of patients with candidemia are negative\textsuperscript{13,14}. iii) Tissue biopsies are often precluded due to the risk of bleeding in the setting of thrombocytopenia, coagulopathy in those with liver disease or hemodynamic instability in critically ill patients. A delay in diagnosis in patients with invasive fungal infection results in higher mortality\textsuperscript{15,16}. Thus, there is an unmet need for novel, rapid, cost-effective, noninvasive diagnostic methods in the field.

Cell-free DNA (cfDNA) technology has been used successfully in noninvasive prenatal testing, organ transplant rejection screening, and oncology liquid biopsies\textsuperscript{17–22}. In recent years, this technology has been developed for use in infectious disease diagnostics\textsuperscript{23,24}. Detection of microbial cfDNA by next generation sequencing (NGS) is an accurate and precise way of identifying and quantifying pathogens\textsuperscript{25}. The Karius\textsuperscript{®} Test relies on sequencing of microbial cfDNA circulating in plasma to identify over 1,000 pathogens, including bacteria, viruses and fungi, from a 5 ml blood sample\textsuperscript{25}. This novel diagnostic tool has been recently validated in a study showing that microbial cfDNA NGS identified 94% of microbes identified by conventional blood culture in patients with sepsis\textsuperscript{25} and has excellent correlation with quantitative PCR testing in patients with cytomegalovirus (CMV)\textsuperscript{21,25}.

Recent reports indicate that NGS measuring microbial cfDNA is useful in the diagnosis of cases of Streptococcus pneumoniae-related hemolytic urticary syndrome, Coxiella burnettii endocarditis, invasive Mycobacterium chimaera infection, Nocardia cyriacigeorgica pneumonia, Capnocytophaga canimorsus sepsis, M. tuberculosis complex and M. haemophilum infections, M. bovis aortitis; Candida spp., Aspergillus spp., non-Aspergillus molds invasive infections; Pneumocystis jiroveci pneumonia (PJP), Toxoplasma gondii infection and choioamnionitis, among others\textsuperscript{21,26–31}. Among 21 patients with culture-positive infective endocarditis, cfDNA NGS identified the same organism as blood cultures in 20 patients (95% sensitivity) and additionally identified Enterococcus faecalis in one out of the three patients with definitive culture-negative endocarditis\textsuperscript{34}. Of note, in this study the cfDNA NGS test identified pathogens causing endocarditis in patients pre-treated with antibiotics up to 30 days prior to initial sample collection.

Here we evaluated the clinical utility of NGS for detection of microbial cfDNA in plasma in a cohort of ten patients receiving chemotherapy or transplants with episodes of febrile neutropenia, sepsis or documented infection.

Methods
Study subjects
Adult patients followed at the Sylvester Comprehensive Cancer Center were enrolled between July 31 and October 2, 2018. Inclusion criteria were: i) age >18 years old; ii) patients must have received chemotherapy or transplant; and iii) must have had a febrile illness or documented infection (e.g., positive blood cultures, clinical/radiographic evidence of pneumonia). There were no exclusion criteria. In this proof-of-concept study, most of the patients had an established diagnosis of infection prior to NGS testing. The study was approved by the University of Miami Institutional Review Board (IRB approval #20080899), consistent with principles in the Declaration of Helsinki. Each participant provided written informed consent for their inclusion in the study. No sample size calculation was done; instead the number of patients enrolled was entirely dependent on the number of cfDNA kits made available for the pilot study.

Sample collection and processing
Blood samples (5 mL) were collected in BD vacutainer plasma preparation tubes. Samples were collected at the time of suspected or confirmed infection diagnosis. 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\textsuperscript{®}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\textsuperscript{24}. The quantity for each organism identified was expressed in Molecules Per Microliter (MPM), the number of DNA sequencing reads for the reported organism present per microliter of plasma.

The Karius\textsuperscript{®} Test
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, release GRCh38.p7, and NCBI, U.S. NLM,
Microbial Genomes, respectively). 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 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 text18 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 the NCBI 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 10 min, 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 Bio-tek Mag-Bind® cfDNA kit; catalog number M3298-01, 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

Background patient information

The characteristics of the patients studied are presented in Table 1. The median age was 56 years (range, 20–65) with 60% of participants males. Except for a kidney transplant recipient, all other patients had underlying hematological malignancy and/or had received an HCT. All but one (patient #2) were admitted in the hospital at the time of clinical evaluation. All the patients were receiving antimicrobials at the time of plasma sample collection. Three patients had neutropenia (absolute neutrophil count <500/μL) at the time of febrile illness. All febrile patients had blood cultures collected within 24 hours of plasma sample collection for NGS.

Results of NGS for detecting microbial cfDNA

The kidney transplant recipient had an Aspergillus fumigatus deep-seated abdominal abscess, and Aspergillus cfDNA levels, although detected in plasma, were below the positive reporting threshold. However, among patients with hematological malignancy in whom a microbiological diagnosis was established (n=5), cfDNA NGS testing correlated with other methods in all cases (100% sensitivity). This included patients with proven/probable invasive aspergillosis, PJP, Stenotrophomonas maltophilia bacteremia, CMV and adenovirus viremia. Among four patients with hematological malignancy with negative standard laboratory testing, the NGS test identified causes of bacterial sepsis in two patients (Table 1), both of whom had a compatible clinical scenario and experienced good clinical response to antibiotic therapy with resolution of fever and hypotension.

Discussion

Here we report our experience using cfDNA NGS in the evaluation of immunocompromised patients—predominantly those with hematological malignancy—with febrile illness or documented invasive infections. The study cohort included a heterogeneous group of clinical scenarios, including deep-seated pyogenic abdominal infection, pulmonary nodules/pneumonia, neutropenic fever, and septic shock. The results of this proof-of-concept study, where most of the patients had an established diagnosis of infection prior to NGS testing, complement recent reports studying the use of cfDNA NGS in immunocompromised hosts.
<table>
<thead>
<tr>
<th>Patient</th>
<th>Age, gender</th>
<th>Underlying disease</th>
<th>Clinical scenario</th>
<th>Sample from CVC</th>
<th>Days of antibiotics/antifungals prior to blood draw</th>
<th>Conventional diagnostic method results</th>
<th>Microbial cfDNA pathogen results</th>
<th>MPM</th>
<th>Reference values</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>65F</td>
<td>Kidney transplant</td>
<td>Pyogenic intra-abdominal infection</td>
<td>No</td>
<td>18/182</td>
<td>Aspergillus fumigatus detected by PCR and culture in abdominal fluid</td>
<td>Negative (Aspergillus fumigatus)</td>
<td>15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10</td>
<td>No&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>21M</td>
<td>NHL, HCT day +342</td>
<td>Mediastinal lymphadenopathy</td>
<td>No</td>
<td>0/8</td>
<td>Negative fungal serologies and antigens BAL and lymph node tissue cultures negative</td>
<td>Negative</td>
<td>15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10</td>
<td>No&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>20M</td>
<td>AML, HCT day +9</td>
<td>Neutropenic fever, diarrhea</td>
<td>Yes</td>
<td>8/2</td>
<td>CMV detected &lt;137 IU/mL (subsequently peaked at 2,621 IU/mL) Blood cultures and C. difficile PCR negative</td>
<td>Cytomegalovirus</td>
<td>108&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10</td>
<td>Yes</td>
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<tr>
<td>4</td>
<td>64F</td>
<td>B-ALL MMUD day +291</td>
<td>Fever, cough, lung mass</td>
<td>Yes</td>
<td>6/5</td>
<td>Pneumocystis jirovecii BAL PCR+</td>
<td>Pneumocystis jirovecii</td>
<td>263&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10</td>
<td>Yes</td>
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<td>5</td>
<td>37M</td>
<td>Relapsed DLBCL after CAR-T</td>
<td>Neutropenic fever, weakness, diarrhea, cough</td>
<td>Yes</td>
<td>21/5</td>
<td>Adenovirus 480 copies/mL (subsequently peaked at 2,600 copies/mL)</td>
<td>Adenovirus</td>
<td>845&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10</td>
<td>Yes</td>
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<td>6</td>
<td>56M</td>
<td>AML, MMUD day +290</td>
<td>Pulmonary nodules (recently diagnosed IA) admitted with SOB</td>
<td>Yes</td>
<td>6/21</td>
<td>CMV detected &lt;137 IU/mL (subsequently peaked at 440 IU/mL) Repeat BAL negative</td>
<td>Cytomegalovirus</td>
<td>93&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10</td>
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<td>7</td>
<td>44M</td>
<td>DLBCL</td>
<td>FEVERS, pulmonary nodules</td>
<td>Yes</td>
<td>3/3</td>
<td>Blood cultures negative</td>
<td>Rothia mucilaginosa</td>
<td>20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;10</td>
<td>No</td>
</tr>
<tr>
<td>Patient</td>
<td>Age, gender</td>
<td>Underlying disease</td>
<td>Clinical scenario</td>
<td>Days of antibiotics/antifungals prior to blood draw</td>
<td>Conventional diagnostic method results</td>
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<td>Reference values</td>
<td>Correlation</td>
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<tr>
<td>8</td>
<td>60F</td>
<td>MDS, HCT day+160, GI-GVHD</td>
<td>Septic shock, multi-organ failure</td>
<td>Yes 15/10</td>
<td>Blood cultures negative</td>
<td>Escherichia coli</td>
<td>2,492</td>
<td>&lt;17</td>
<td>No</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Lactobacillus rhamnosus</td>
<td>Torque teno virus</td>
<td>308</td>
<td>&lt;10</td>
<td></td>
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<tr>
<td>9</td>
<td>55F</td>
<td>Multiple myeloma</td>
<td>Pneumonia</td>
<td>Yes 2/0</td>
<td>Negative BAL studies</td>
<td>Negative</td>
<td></td>
<td></td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>10</td>
<td>58M</td>
<td>AML</td>
<td>Neutropenic fever, pulmonary nodules, sepsis</td>
<td>Yes 120/129</td>
<td>S. maltophilia in blood cultures Pan-Aspergillus PCR+ in BAL Serum galactomannan+</td>
<td>Stenotrophomonas maltophilia Aspergillus oryzae Staphylococcus epidermidis</td>
<td>236,594</td>
<td>&lt;83</td>
<td></td>
<td>Yes</td>
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<td></td>
<td>11,533</td>
<td>9,673</td>
<td></td>
<td>10</td>
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<td></td>
</tr>
</tbody>
</table>

- Refers to empiric or targeted therapy only. It does not include days of antimicrobial prophylaxis.
- Blood cultures were obtained within 24h of plasma sample for NGS in all patients and resulted as negative unless specified otherwise in the table.
- Reference value is the 97.5th percentile in self-reported healthy adults for whom the Karius® Test was performed.
- Correlation between Karius® Test and standard laboratory methods.
- Aspergillus fumigatus reads were present in the raw data but below the threshold for a positive test result.
- Initial cfDNA testing performed 7 weeks prior had only identified S. epidermidis and EBV. At that time, BAL and transbronchial biopsy results were unrevealing.

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; BAL, bronchoalveolar lavage; CAR-T, chimeric antigen receptor-modified T-cell immunotherapy; cfDNA, cell-free DNA; CMV, cytomegalovirus; CVC, central venous catheter; DLBCL, diffuse large B cell lymphoma; GI-GVHD, gastrointestinal graft-versus-host disease; HCT, hematopoietic cell transplantation; F, female; M, male; MPM, molecules per microliter; NGS, next-generation sequencing; NLH, Non-Hodgkin lymphoma; SOB, shortness of breath.
In a recent study of 55 patients with neutropenic fever, cfDNA testing had positive agreement with conventional blood cultures in 9 of 10 patients in whom blood cultures identified a causative organism of sepsis. Using clinical adjudication by three infectious diseases specialists, cfDNA NGS had a sensitivity of 85.4% (41/48) and specificity of 100% (7/7). This, thus, this test is a promising diagnostic tool in neutropenic fever, a clinical scenario where conventional work up fails to identify an etiological agent in a majority of cases. Another study evaluated 40 patients with prolonged neutropenia and fever (>96h) despite administration of antibiotics for suspected fungal infection (the authors excluded patients who had received antifungal therapy for >3 days); in this study cfDNA NGS identified fungal pathogens including *Aspergillus fumigatus*, *Rhizopus spp.*, and *Candida albicans*, *Candida glabrata* and *Pneumocystis jiroveci*. In that report, there was one case where the plasma sample was obtained after at least 15 days of anti-*Aspergillus* therapy, and NGS testing did not identify the causal organism of invasive fungal infection. Similarly, for the kidney transplant patient reported here with invasive aspergillosis, in whom *Aspergillus fumigatus* cfDNA levels in plasma were detected below the reporting threshold, several months of antifungal therapy had been administered prior to the time of plasma collection. Thus, prolonged antifungal therapy prior to sample collection (e.g., >7-14 days) might interfere with detection of fungal DNA.

Although NGS has been used for screening of allograft rejection in solid organ transplant recipients, there is limited data with the use of NGS for diagnosis of infections in this population. A recent study demonstrated strong correlation between clinical test results and cfDNA derived from CMV in a cohort of lung transplant recipients. In addition, cfDNA revealed undiagnosed cases of infection with microsporidia and pathogenic viruses, including adenovirus and human herpesvirus 6 among lung transplant patients.

Recently, Fung *et al.* reported three patients who received allogeneic HCT transplant in whom NGS cfDNA facilitated the diagnosis of an uncommon presentation of *Chlamydia trachomatis* and recurrent and metastatic complications of *Staphylococcus aureus* bacteremia before standard microbiology.

The fact that in our cohort cfDNA NGS testing identified the cause of febrile illness in two patients with culture-negative sepsis who had a compatible clinical syndrome and responded well to antibiotic therapy supports the notion that NGS testing can be a useful diagnostic tool, particularly when conventional blood cultures are negative. The Karius® Test pathogen-specific reference ranges have been established using cfDNA levels from healthy donors. Patient #8 had detectable levels of Torque teno virus, which belongs to *Anelloviridae* family and is considered to lack pathogenic potential; this suggests the possibility that cfDNA NGS might on occasion yield detection of members of the commensal microbiota or viroma. To our surprise, however, even though many of the patients tested had mucosal barrier damage (e.g., mucositis) allowing for bacterial translocation from the gut, the Karius® Test did not show a non-specific gut flora signal. The test was negative in patients in whom we failed to establish a microbiological diagnosis for their febrile illness, and when positive, typically correlated with conventional laboratory testing. Whether the currently defined cfDNA thresholds are optimal for identifying and quantifying pathogens of clinical relevance in highly vulnerable immunocompromised hosts will require further study. Importantly, the turnaround time for results was consistently within 48 hours, which is quite rapid considering that samples were shipped overnight from our institution located in Florida to the Karius Inc. laboratory in California.

Lack of control group, small number of patients and the heterogeneity of the cohort in terms of underlying diseases and causes of immunosuppression represent major limitations of this report. Larger clinical trials evaluating plasma NGS in patients with cancer and undergoing transplant are ongoing (NCT03226158, NCT03262584, NCT02912117, NCT02804464). Until larger cohort data becomes available, our observations suggest that detection of microbial cfDNA using NGS is valuable for the rapid noninvasive diagnosis of infectious complications following chemotherapy or transplantation.

**Conclusion**

Our data, along with a number of recent reports, support the clinical utility of the measurement of microbial cfDNA in peripheral blood using NGS for rapid noninvasive diagnosis of infections in immunocompromised hosts. As with other novel laboratory diagnostics used in clinical practice, the results of cfDNA NGS technology need to be interpreted with caution and in conjunction with other laboratory, radiological and clinical findings. Larger studies are needed to validate these findings.

**Data availability**

**Underlying data**

Microbial cfDNA NGS for Rapid Noninvasive Diagnosis of Infectious Diseases in Immunocompromised Hosts, BioProject accession number PRJNA554271.

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