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## CASE REPORT

### Utility of Whole-Genome Next-Generation Sequencing of Plasma in Identifying Opportunistic Infections in HIV/AIDS

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#### Abstract:

#### Background:

AIDS-associated Opportunistic Infections (OIs) have significant morbidity and mortality and can be diagnostically challenging, requiring invasive procedures as well as a combination of culture and targeted molecular approaches.

#### Objective:

We aimed to demonstrate the clinical utility of Next-generation Sequencing (NGS) in pathogen identification; NGS is a maturing technology enabling the detection of miniscule amounts of cell-free microbial DNA from the bloodstream.

#### Methods:

We utilized a novel Next-generation Sequencing (NGS) test on plasma samples to diagnose a series of HIV-associated OIs that were diagnostically confirmed through conventional microbial testing.

#### Results:

In all cases, NGS test results were available sooner than conventional testing. This is the first case series demonstrating the utility of whole-genome NGS testing to identify OIs from plasma in HIV/AIDS patients.

#### Conclusion:

NGS approaches present a clinically-actionable, comprehensive means of diagnosing OIs and other systemic infections while avoiding the labor, expense, and delays of multiple tests and invasive procedures.

**Keywords:** Human immunodeficiency virus, Acquired Immune Deficiency Syndrome, Opportunistic infections, Next-generation sequencing, Cell-free DNA, *Toxoplasma gondii*, Toxoplasmosis, *Mycobacterium avium* complex, *Mycobacterium tuberculosis*, *Cryptosporidium*.

#### Article History

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## 1. BACKGROUND

Despite advancements in Highly-active Antiretroviral Therapy (HAART) and overall survival in HIV/AIDS, Opportunistic Infections (OIs) remain a significant cause of AIDS-associated morbidity and mortality, particularly in the setting of advanced disease and delayed or limited access to

care [1]. Many OIs remain diagnostically challenging, requiring invasive procedures for tissue/fluid sampling as well as a combination of culture and targeted molecular approaches that can be laborious, expensive and limited by the sensitivity/specificity of individual assays. Next-generation Sequencing (NGS) is a maturing technology enabling detection of miniscule amounts of genetic material [2] that has demonstrated clinical utility in the diagnosis of fetal chromosomal anomalies [3], solid organ transplant rejection [4], and more recently pathogen identification [5 - 10]. We

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describe our experience in the diagnosis of AIDS-associated OIs at a single institution with a novel, noninvasive NGS test that detects pathogen cell-free DNA (cfDNA) from plasma samples [5 - 7]. Cases included in this series are summarized in the accompanying Table and described below.

Methods for sample preparation and plasma NGS for microbial cfDNA in the present series have been previously described [5, 6] and validated in terms of accuracy, precision, bias and robustness [5]. Briefly, blood was collected in a Plasma Preparation Tube (PPT, BD Biosciences), centrifuged at 1100 RCF for 10 minutes and then shipped to a CLIA-certified, CAP-accredited laboratory for analysis (Karius, Inc., Redwood City, CA), where cfDNA was extracted from plasma. For NGS, sequencing libraries were constructed and control samples were processed alongside test samples in every batch. Batched libraries were multiplexed and sequenced on Illumina NextSeq500 sequencers. Samples generated, on average, approximately 24 million sequencing reads each. After removing human reads, remaining sequences were aligned using BLAST+ [11] to a proprietary curated microorganism database allowing detection of over 1000 microbial pathogens including DNA viruses, bacteria, fungi and other eukaryotes.

Several filtering algorithms were used to mitigate false-positive results, including removal of environmental contaminants and cross-reactivity with other microorganisms [5]. Subsequently, organisms present above a predefined significance threshold were reported. Analysis methods have been described in detail elsewhere [5, 6].

## 2. CASE PRESENTATIONS

### 2.1. Case 1

#### 2.1.1. CNS Toxoplasmosis

A 49-year-old man presented with one week of left hand weakness and two days of progressive headache, nausea, and vomiting. Notably, two weeks prior at an outside hospital the patient was found to have an enhancing intra-cerebral mass, with initial pathology from biopsy concerning for high-grade glioma. Repeat head CT on the present admission showed no new findings. On hospital day 4, the patient became more somnolent, and CT showed increased cerebral edema which improved with medical management. On day 10, brain MRI performed to evaluate for primary malignancy revealed

**Table 1. Summary of HIV-associated opportunistic infections identified by NGS\*.**

-	Case 1 – CNS Toxoplasmosis	Case 2 – Disseminated MAC	Case 3 – TB Lymphadenitis	Case 4 – Microsporidiosis
Demographics				
Age (years)	49	23	56	45
Gender	M	M	M	M
HIV/AIDS information				
CD4+ cell count (cells/ $\mu$ L)	4	9	25	339
HIV-1 viral load (copies/mL)	197,000	809,000	527,000	221,000
Localization of infection	CNS	Disseminated	Disseminated	Gastrointestinal
Other microbiology tests ordered	- Blood cultures for bacteria, fungi, AFB - Blood TB T-spot - Blood antigen for <i>Strongyloides</i> , <i>Cryptococcus</i> , <i>Histoplasma</i> and <i>Toxoplasma</i> - BAL cultures for bacteria, fungi, AFB, <i>Mycoplasma</i> and <i>Legionella</i> ; PCR for <i>Chlamydia pneumoniae</i> - CSF cultures, PCR for <i>Toxoplasma</i> , JC virus, EBV, TB - Brain tissue pathology staining for multiple organisms	- Blood cultures for bacteria, fungi, AFB - Bone marrow biopsy cultures and staining for bacteria, viruses, fungi, AFB	- Blood cultures for bacteria, fungi, AFB - BAL cultures for bacteria, viruses, fungi, AFB, <i>Mycoplasma</i> and <i>Legionella</i> ; PCR for <i>Chlamydia pneumoniae</i> - Lung tissue cultures and staining for bacteria, viruses, fungi, AFB - CSF cultures, VDRL and antigens for <i>Cryptococcus</i> and WNV - Lymph node biopsy tissue cultures and staining for bacteria, fungi, AFB	- CSF cultures, multiple viral PCRs, and AFB stain/culture - Stool ova and parasites - GI pathogen PCR panel
Positive microbiology test(s)	Blood <i>Toxoplasma</i> IgG; CSF <i>Toxoplasma</i> PCR; Brain tissue <i>Toxoplasma</i> stain	Bone marrow biopsy AFB culture; AFB blood culture	Lung tissue AFB culture; lymph node tissue x2 AFB stain and culture	GI pathogen panel positive Norovirus and <i>Cryptosporidium</i>
NGS result(s)	<i>Toxoplasma gondii</i> ; Cytomegalovirus; <i>Nocardia farcinica</i> ; <i>Enterococcus faecalis</i> and <i>Enterococcus faecium</i>	<i>Mycobacterium avium</i> complex	<i>Mycobacterium tuberculosis</i> ; EBV	<i>Enterocytozoon bienewisi</i>

\*NGS, next-generation sequencing; CNS, central nervous system; AFB, acid-fast bacilli; TB, tuberculosis (*Mycobacterium tuberculosis*); BAL, bronchoalveolar lavage; EBV, Epstein-Barr Virus; CSF, cerebrospinal fluid; MAC, *Mycobacterium avium* complex; WNV, West Nile Virus; GI, gastrointestinal.

ring-enhancing lesions in the left cerebral hemisphere and right cerebellum. Subsequent HIV testing was positive (CD4+ cell count 4 cells/mL, HIV-1 viral load 197,000 copies/mL) and on day 11 empiric trimethoprim-sulfamethoxazole was initiated for possible CNS toxoplasmosis. Chest CT showed bilateral pulmonary infiltrates, with subsequent bronchoscopic workup unrevealing (Table 1). Blood cultures and antigen tests for *Strongyloides* (stool), *Cryptococcus* (blood) and *Histoplasma* (urine) were also negative. Blood Cytomegalovirus (CMV) PCR was positive. *Toxoplasma* serum IgG was positive with negative IgM. Lumbar Puncture (LP) could not be performed safely due to the risk of cerebral herniation.

Minimal clinical improvement was seen after one week of hospitalization, and a repeat brain MRI suggested disease progression. Treatment was progressively escalated to pyrimethamine, sulfadiazine, and leucovorin for CNS toxoplasmosis (day 28) in addition to empiric treatment for bacterial infection, CMV, and Tuberculosis (TB). Plasma NGS testing sent on day 28 was positive on day 30 for *Toxoplasma gondii*, *Nocardia farcinica*, CMV, *Enterococcus faecalis* and *Enterococcus faecium*. Pathology slides obtained from the outside hospital during the patient's prior admission were stain-positive for *Toxoplasma*. On day 34, HAART was initiated and on day 39 steroids were escalated and an extraventricular drain (EVD) placed for mental deterioration and possible paradoxical Immune Reconstitution Inflammatory Syndrome. Cerebrospinal Fluid (CSF) obtained from the EVD was subsequently positive for *Toxoplasma gondii* PCR. The patient gradually improved and was discharged on day 56 with prolonged treatment for CNS toxoplasmosis until his CD4+ count recovered to >200 cells/mL.

## 2.2. Case 2

### 2.2.1. Disseminated *Mycobacterium Avium* (MAC)

A 23-year-old male with intravenous drug use and known HIV/AIDS (CD4+ cell count 9 cells/mL, HIV-1 viral load 809,000 copies/mL) was admitted two weeks after starting HAART with intermittent fever and night sweats without localizing symptoms. Labs demonstrated mild transaminitis and elevated Alkaline Phosphatase (ALP). Initial blood cultures were unrevealing. CT chest, abdomen and pelvis showed multiple prominent mediastinal, abdominal, and pelvic Lymph Nodes (LNs).

After 9 days of persistent fever, the patient was started on empiric MAC therapy. A plasma NGS test was sent, detecting *Mycobacterium Avium* Complex (MAC) and CMV two days later. Bone marrow biopsy demonstrated granulomas with negative AFB and fungal stains. Blood CMV PCR was negative, but AFB blood cultures grew MAC several days after the NGS result. Bone marrow samples eventually grew MAC as well. The patient defervesced on appropriate MAC therapy and was discharged.

## 2.3. Case 3

### 2.3.1. *Mycobacterium Tuberculosis* (MTB) Lymphadenitis

A 56-year-old male with untreated HIV/AIDS (CD4+ cell

count 25 cells/mL, HIV-1 viral load 527,000 copies/mL) presented with 6 weeks of worsening fatigue, confusion, imbalance, headaches, abdominal pain, diarrhea, and anorexia. On admission, he was febrile, tachycardic, and cachectic, with initial labs showing leukopenia, anemia and elevated creatinine. He denied sick contacts, travel, animal exposures, or new foods, and was last sexually active six months prior with consistent condom use. Chest x-ray was unremarkable and CT abdomen and pelvis showed reactive LNs in the abdomen and retroperitoneum. The patient was started on vancomycin and cefepime but continued having high fevers, so ampicillin and acyclovir were added. Diagnostic workup included negative blood cultures, negative fecal leukocytes, and normal CSF studies (Table 1). Brain imaging showed no acute abnormalities. Empiric treatment was initiated for suspected disseminated MAC infection. CT chest showed multiple pulmonary nodules and patchy airspace disease. Bronchoscopy and tissue pathology revealed granulomas, but multiple diagnostic studies were negative (Table 1).

Plasma NGS testing sent on day 4 was positive on day 10 for MTB and EBV. Retroperitoneal LN biopsy on day 4 showed acute necrotizing lymphadenitis with positive AFB stain. Isoniazid and pyrazinamide were added for TB treatment. The patient improved and was discharged on day 17 with 4-drug TB treatment, HAART and relevant OI prophylaxis. AFB cultures of LN and respiratory samples eventually became positive for MTB following hospital discharge 21 and 26 days after collection, respectively. The patient's CD4+ count increased from 25 to 93 cells/mm<sup>3</sup> and viral load decreased from 527,000 to 365 copies/mL on appropriate therapy.

## 2.4. Case 4

### 2.4.1. *Microsporidiosis*

A 45-year-old man without significant medical history presented with 6-8 weeks of chronic diarrhea unresponsive to empiric metronidazole treatment. He was newly diagnosed with HIV (CD4+ cell count 339 cells/mL, HIV-1 viral load 221,000 copies/mL) with mild transaminitis and elevated ALP. He experienced intermittent fevers during hospitalization. CT chest, abdomen, and pelvis revealed diffuse lymphadenopathy. LP showed CSF WBC count 160 cells/mm<sup>3</sup> with lymphocyte predominance (91%), protein 84 mg/dL, and glucose 37 mg/dL. Multiple CSF studies were negative (Table 1). Stool ova/parasite examination was negative for *Cyclospora*, *Isospora* and *Cryptosporidia*. A gastrointestinal pathogen stool PCR panel tested positive for Norovirus and *Cryptosporidium*. Plasma NGS testing was positive for *Enterocytozoon bieneusi*, a cause of microsporidiosis, on day 6. It should be noted that Norovirus is an RNA pathogen and as such, would not be detected by plasma NGS testing for cfDNA. Also, the GI PCR panel used does not test for *Enterocytozoon* or other Microsporidia. The detection of *Enterocytozoon* by NGS was felt by the treating physician to be consistent with the detection of *Cryptosporidium* by stool PCR testing, as Microsporidia/Cryptosporidia co-infections can be seen in up to 81% of HIV/AIDS patients with chronic diarrhea [12]. The patient was started on nitazoxanide to treat both

*Cryptosporidium* and *Microsporidium* [13] and symptoms resolved with treatment.

## DISCUSSION AND CONCLUSION

OIs are often the initial presentation for the estimated 15% of HIV-positive U.S. adults who are unaware of their HIV status [14]. Timely diagnosis remains critical to improving survival and preventing long-term sequelae. Diagnosis of CNS OIs can be challenging due to limited availability and/or sensitivity of the array of simultaneous diagnostic tests typically used, including CSF microscopy, antibody/antigen and targeted PCR testing, serologies for specific pathogens, neuroimaging and brain biopsy [15, 16]. Similarly, diagnosis of mycobacterial infections such as MTB or disseminated MAC relies on a combination of radiologic imaging, skin or blood tests, and tissue analysis including microscopy, AFB culture, and PCR. These methods are time-consuming and costly, have varying sensitivity and specificity, and/or necessitate invasive tissue sampling for definitive diagnosis [17, 18].

The advantages of NGS approaches to infectious disease diagnosis include rapidity, accuracy, breadth of detection, and avoidance of laborious, costly and/or invasive procedures. We describe 4 cases of HIV-associated OIs where diagnostic uncertainty along with critical illness requiring aggressive management prompted the use of a novel high-sensitivity NGS platform that provided rapid, unbiased detection of cell-free pathogen DNA from a plasma sample. Results from plasma NGS were available faster than conventional diagnostics and were consistent with other test results (Table 1) as well as the clinical scenario.

Nonetheless, a number of challenges must be overcome in order to apply NGS approaches to clinical infectious disease practice. Although strong pathogen nucleic acid signal is present in cell-free plasma, there is a significantly higher abundance of human DNA. To overcome this challenge, an enrichment protocol was developed that allows for efficient detection of pathogen signal relative to the background. Furthermore, a high-quality microorganism database is essential to support a comprehensive and accurate assay. Reference microorganism sequences originating from public databases are known to be both incomplete and inaccurate. To address potential errors stemming from inaccuracies in sequence data, a proprietary database was developed that 1) includes only reference sequences with indicators of high-quality assembly (*e.g.* completeness, N50 score, *etc.*); 2) is continuously curated to minimize human cross-reactivity as well as cross-reactivity between pathogens; and 3) is screened to mitigate contamination with sequences from human or other organisms. The clinical challenges of utilizing NGS include distinguishing colonization from true infection which can affect specificity. In the cases presented, polymicrobial results could be difficult to interpret. However, when considering the high pretest probability for particular OIs, the relevant and causative pathogens were readily discerned. Finally, NGS testing for clinical care requires sophisticated instrumentation and personnel expertise alongside robust procedures and protocols that will mitigate the risks of contamination and errors in specimen handling. In the present series, NGS was

performed at a CAP-accredited and CLIA-certified reference laboratory which currently provides validated commercial NGS testing for hospitals.

The advantages of using cell-free DNA for non-invasive diagnostic testing are already being realized in prenatal diagnosis of genetic disorders [3], cancer diagnostics [19], and transplant rejection monitoring [4], and are more recently being realized in the field of infectious diseases [5 - 10]. This is the first case series describing the utility of NGS on cfDNA for diagnosis of OIs in advanced HIV/AIDS patients. We propose that genome-wide approaches to pathogen identification are now clinically relevant and actionable, and offer distinct advantages over traditional diagnostic methods in feasibility, avoidance of invasive procedures, cost-effectiveness, and clinical outcomes. Further studies will seek to validate and document the cost, time, and impacts on morbidity/ mortality.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

## HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

## CONSENT FOR PUBLICATION

Not applicable.

## CONFLICT OF INTEREST

The authors S.C. Dalai and D.K. Hong are employees of Karius, Inc. All other authors have no conflicts of interest to report.

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