Next-generation sequencing in neuropathologic diagnosis of infections of the nervous system

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Supplemental data at Neurology.org/nn

ABSTRACT

Objective: To determine the feasibility of next-generation sequencing (NGS) microbiome approaches in the diagnosis of infectious disorders in brain or spinal cord biopsies in patients with suspected CNS infections.

Methods: In a prospective pilot study, we applied NGS in combination with a new computational analysis pipeline to detect the presence of pathogenic microbes in brain or spinal cord biopsies from 10 patients with neurologic problems indicating possible infection but for whom conventional clinical and microbiology studies yielded negative or inconclusive results.

Results: Direct DNA and RNA sequencing of brain tissue biopsies generated 8.3 million to 29.1 million sequence reads per sample, which successfully identified with high confidence the infectious agent in 3 patients for whom validation techniques confirmed the pathogens identified by NGS. Although NGS was unable to identify with precision infectious agents in the remaining cases, it contributed to the understanding of neuropathologic processes in 5 others, demonstrating the power of large-scale unbiased sequencing as a novel diagnostic tool. Clinical outcomes were consistent with the findings yielded by NGS on the presence or absence of an infectious pathogenic process in 8 of 10 cases, and were noncontributory in the remaining 2.

Conclusions: NGS-guided metagenomic studies of brain, spinal cord, or meningeal biopsies offer the possibility for dramatic improvements in our ability to detect (or rule out) a wide range of CNS pathogens, with potential benefits in speed, sensitivity, and cost. NGS-based microbiome approaches present a major new opportunity to investigate the potential role of infectious pathogens in the pathogenesis of neuroinflammatory disorders.

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GLOSSARY

EBV = Epstein-Barr virus; FIRES = febrile infection-related epilepsy syndrome; JCV = JC polyomavirus; NGS = next-generation sequencing; PML = progressive multifocal leukoencephalopathy.

Ascertaining of the etiology of inflammatory disorders of the CNS represents a major challenge in the clinical setting, as more than 50% of cases go undiagnosed.1 Next-generation sequencing (NGS) and metagenomics present a major new opportunity to investigate the potential role of infection in the pathogenesis of neuroinflammatory disorders. This technology can provide a view of the transcriptome of the host tissue as well as capture microbial genomes (i.e., bacteria, fungi, and viruses) that reside in the tissue niche.2–4 Until recently, most sequence-based pathogen identification studies have focused on targeted capture of the 16S rRNA gene that is exclusive to prokaryotes. Deep sequencing of total DNA or RNA provides an unbiased approach that can detect even rare components of the microbiome.5 This strategy has recently been used to diagnose cases of encephalitis and meningitis by known and novel pathogens,6–15

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Sequencing-based diagnosis shows the microorganism indicated by sequencing as a possible cause of infection. Detailed read counts for all bacterial and viral species, as well as the number of fungal reads, are found in tables e-1 through e-3.

**METHODS** Patients, biopsy handling, and sequencing. CNS tissues were obtained prospectively from biopsies performed during diagnostic assessment of 10 patients with neuroinflammatory disorders (table 1). Fresh frozen tissues from 8 cases were sequenced immediately after biopsy and 2 other samples were from paraffin-processed tissues (e-Methods at Neurology.org/nn).

**RESULTS** Sequencing data. The sequenced biopsies (appendix e-1) generated between 8.3 and 29.1 million reads per sample (table 1). The vast majority of reads in all cases were human, as expected (because the human genome is approximately 1,000 times larger than most bacterial genomes, a mixture with equal numbers of human and bacterial cells will yield 99.9% human DNA). After filtering to remove common contaminants and vector sequences, we then ranked the microbial species and considered only those that ranked among the top 3 species as possible infectious agents but other than these isolated cases, the utility of NGS for clinical or pathologic diagnosis has yet to be established.

We report a pilot prospective study of the use of unbiased NGS to assist in the evaluation of brain biopsies in a series of patients with neuroinflammatory disorders suspected to be associated with infections. We applied NGS and a new computational analysis pipeline for identifying pathogen species based on short NGS reads, as short as 100 base pairs (bp) in length.16

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confirmed. to indicate the 3 samples for which the sequence-based diagnosis was independently species in that sample, ranging from blue (1%) to red (100%). Green boxes across the bot-

All microbial species that explain 10% or more of microbial reads (excluding contaminants) in

Cases with a high degree of diagnostic confidence and positive pathogen identification. Patient PT-8 (pathogen: Mycobacterium tuberculosis). A 67-year-old woman with osteomyelitis and lung disease presented with multifocal brain and spinal lesions (appendix e-2). Brain MRI showed progression of multiple nodular enhancing lesions throughout the brain (figure 2). Biopsies from a perilesional (S1) and a nodular brain lesion (S2) were obtained for pathology, microbiology, and NGS studies. Two DNA sequencing runs yielded 15 M reads from sample S1 and 14 M reads from sample S2. These runs yielded the fewest microbial reads of any of the patients in our study: 18 and 22 bacterial reads, and only 1 to 6 viral and fungal reads, respectively, for samples S1 and S2. Nonetheless, a clear finding emerged for sample S2: 15 reads from M tuberculosis. Despite the small absolute number of reads, this species explained 68% of the bacterial reads detected. We manually confirmed the sequence assignments using BLAST17 to align them against the NCBI nt database. We then realigned all reads against one specific genome, M tuberculosis 7199-99 (accession NC_020889.1), using Bowtie2 with sensitive local alignment settings.18 This procedure yielded 34 reads that were randomly distributed along the M tuberculosis genome. Pathology studies of the corresponding S2 sample showed necrotizing granulomas although microbiologic studies failed to identify any microorganism (figure 2). The patient responded rapidly to antituberculous treatment.

Patient PT-5 (pathogen: JC polyomavirus [JCV]). A 52-year-old man had been admitted for evaluation of lower extremity weakness, right hemiparesis, and a simple partial motor seizure. A brain MRI showed a left postcentral frontal cortical and white matter lesion (figure 3A). PCR CSF studies for viruses and bacterial cultures were negative although a pre-

Figure 1 Heatmap shows the top microbial species in each of the 11 samples

All microbial species that explain 10% or more of microbial reads (excluding contaminants) in any sample are shown. Shaded squares indicate the relative proportion of reads from each species in that sample, ranging from blue (1%) to red (100%). Green boxes across the bottom indicate the 3 samples for which the sequence-based diagnosis was independently confirmed.

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A retro-orbital headache after cataract and lens implantation surgeries. The symptoms were followed by decreased vision, diplopia, ophthalmoplegia, and facial numbness. He was diagnosed with Tolosa-Hunt syndrome and treated with dexamethasone. Brain MRIs demonstrated pachymeningeal enhancement localized in the medial aspect of the left middle cranial fossae, left cavernous sinus, and adjacent structures (figure 5A), which worsened over time despite steroid treatment. A biopsy of the dural and skull base mass showed chronic inflammation and fibrosis (figure 5C). Microbiologic studies were negative. NGS studies of the dura biopsy were indeterminate although a high proportion of *Delftia acidovorans* (or possibly *Chryseobacterium taeanense*, which is very closely related to *Delftia*)
Corynebacterium kroppenstedtii reads were seen, higher (45% of nonhuman and noncontaminant reads) than in any other patient (appendix e-2). Although Delftia might have been a contaminant, a combination of antibiotic treatments produced clinical improvement and decreased dural enhancement in brain MRI (figure 5B). It was concluded that the pachymeningeal reaction was possibly associated with a chronic evolving infection possibly triggered by cataract surgery and intraocular lens implantation although no confirmatory studies were obtained to link the potential role of Delftia or other bacteria in the pathogenesis of pachymeningitis.

Patient PT-7 (Fanconi anemia, neurodevelopmental disorder, and cerebritis). A 19-year-old man developed new-onset right-sided weakness and seizures. A brain MRI demonstrated a left-sided brain mass (figure e-1) and a brain biopsy suggested the diagnosis of lymphoma. He was treated with IV and intrathecal dexamethasone via an Ommaya reservoir. The patient deteriorated clinically and was transferred to our institution. A second biopsy of the brain lesion showed chronic inflammation (figure e-1) and coagulative necrosis. Microbiological studies were negative. NGS analysis of the biopsy did not reveal a clear candidate, but did show an unusually high presence of Lactococcus lactis, a common additive in dairy products that rarely causes human infections. A total of 244 reads mapped to the genus Lactococcus, 201 of which were specific to L. lactis cremoris.

However, 1 year later (March 2016), re-analysis of this patient’s data revealed 429 reads from Elizabethkingia, a newly emerging pathogen that caused significant morbidity in a cluster of cases in Wisconsin.20 No more than 2 reads from this species were detected in any other sample. The genomes for this species were unavailable at the time of the original analysis, but 3 genomes are now available: EspB M10, E. anophilis NUHP1, and E. meningoseptica FMS-007. The greatest number of matches in this sample was to EspB M10.

Cases with nonspecific or negative findings that were clinically useful. Sequencing yielded no specific findings to support a diagnosis of infection in 5 cases (table 1). In case PT-3, a 23-year-old woman with status epilepticus following a febrile illness was diagnosed with febrile infection-related epilepsy syndrome (FIRES),21 after extensive microbiologic and NGS studies were negative for pathogens. In
cases PT-4 and PT-9, suspected to have granulomatous inflammation or sarcoidosis based on brain MRI and biopsy findings, NGS showed no evidence of specific infection. Subsequent steroid treatment was associated with improvement in both cases. In 2 cases, neuropathologic studies demonstrated that the disease process was associated with glial tumors despite initial concerns about infections. In 1 patient, PT-6, DNA sequencing identified 15 viral reads mapped to JCV, a finding considered to be incidental, although JCV has been implicated in the pathogenesis of astrocytomas in nonhuman primates.22

DISCUSSION

We describe the power of large-scale unbiased sequencing along with computational analysis as a diagnostic tool to establish the presence or absence of active infection in brain biopsies from patients with neuroinflammatory disorders suspected to be associated with pathogenic microorganisms. With current technology, the minimum time required for sequencing is approximately 1 day, with initial analysis requiring less than an hour after the sequencing is complete. The current clinical standards for neuropathologic diagnosis of infections, which include histologic, immunologic, and selected molecular techniques (e.g., PCR, 16S rRNA, in situ hybridization), have limited power even when specific neurologic infectious disorders are suspected.

In our study, NGS facilitated the identification of a pathogenic agent in 3 patients for whom the presence of such agent was further validated by immunohistologic or molecular techniques. In these 5 patients, standard diagnostic studies had failed to define etiologic agents. In case PT-8, in which *Mtuberculosis* was identified by NGS, the findings of sequencing studies along with brain pathology features of necrotizing granuloma supported treatment that led to clinical improvement. Similarly in cases PT-5 and PT-10, the confidence in the findings of JCV and EBV was strengthened by immunocytochemical and in situ hybridization techniques, which further supported the finding of a pathogenic role of these viruses. Although PCR studies for EBV and JCV in CSF are well-established in clinical practice,

Figure 4  Brain imaging and neuropathologic demonstration of Epstein-Barr virus (EBV) encephalitis in patient PT-10

(A–C) Brain MRI shows a focal area of cortical and subcortical signal intensity abnormality. T1-weighted sequences enhanced with gadolinium show a ring-enhanced lesion with perilesional edema, which was the target for the brain biopsy. (D) Histologic demonstration of a granulomatous and lymphohistocytic inflammation with foci of necrosis (hematoxylin & eosin stain) in patient PT-10. (E) In situ hybridization for EBV-encoded RNA in the brain biopsy tissues, which validates the presence of EBV as it had been established by next-generation sequencing and confirms the diagnosis of EBV encephalitis. FLAIR = fluid-attenuated inversion recovery.
there are specific clinical situations in which brain biopsies are required. For example, the presence of EBV DNA in CSF of immunocompromised patients should be interpreted with caution as EBV may coexist with other opportunistic infections. In case PT-10, for which CSF PCR studies were negative, the finding of EBV by NGS validated by EBV-encoded RNA in situ hybridization helped to establish a diagnosis of EBV-associated encephalitis and also counteracted the brain MRI evidence, which was suggestive of toxoplasmosis. This case shows many similarities to a previous report of EBV-induced brain lesions in a patient following organ transplantation. Similarly, the value of metagenomic sequencing in the diagnosis of PT-5 was demonstrated by the inconclusiveness of 2 prebiopsy qualitative JCV-PCR studies in CSF. Although pathology and PCR techniques might have helped to establish the diagnosis of PML, case PT-5 represents a good proof-of-concept that NGS studies are useful and may provide better characterization of the JCV genome for prognosis purposes.

Although a high degree of certainty could not be achieved in some cases, NGS provided information that facilitated a better understanding of the high or low likelihood of CNS infection in the other 5 patients. In 2 cases, NGS findings were considered indeterminate as suspected microorganisms frequently seen as contaminants were identified, although their pathogenic role could not be confirmed by histology, cultures, or direct pathogen visualization. In these 2 patients, the metagenomic analysis yielded a high number of reads of bacteria identified as environmental contaminants such as D acidovorans and C kroppenstedtii (PT-2), normal commensals of skin, and L lactis cremoris and Elizabethkingia (PT-7). Although these bacteria might have entered the CNS by surgical procedures such as ophthalmologic surgery (PT-2) and Ommaya reservoir (PT-7), the validation that these assumed contaminants were pathogenic organisms by other microbiologic or morphologic methods was difficult to obtain, for which the NGS findings were considered indeterminate. Although these environmental contaminants have been associated with neuropathologic conditions and outbreak of illness, our findings expose one of the limitations in the interpretation of NGS, in that the demonstration that these presumed contaminants were pathogenic organisms would require a detailed validation approach to prove pathogenicity.

Whereas microbiome analysis of brain biopsies was able to identify definite or possible pathogenic microorganisms in 5 of 10 cases, no pathogenic species were identified in the other 5 cases. Negative NGS studies may help support a finding that a brain infection is not present, although the negative predictive value of sequencing assays is not yet known. In case PT-3, the failure to demonstrate a pathogen helped to determine her neurologic disorder was most likely associated with FIRES, a rare condition of uncertain etiology in which infection is suspected. In another 2 cases, PT-4 and PT-9, NGS findings were considered indeterminate as suspected microorganisms frequently recognized as environmental contaminants were identified. In 2 of the 10 patients, the metagenomic analysis yielded a high number of reads of bacteria identified as environmental contaminants such as Lactococcus, normal commensals of skin, and Elizabethkingia. Although these bacteria might have entered the CNS by surgical procedures such as ophthalmologic surgery (PT-2) and Ommaya reservoir (PT-7), the validation that these assumed contaminants were pathogenic organisms by other microbiologic or morphologic methods was difficult to obtain, for which the NGS findings were considered indeterminate. Although these environmental contaminants have been associated with neuropathologic conditions and outbreak of illness, our findings expose one of the limitations in the interpretation of NGS, in that the demonstration that these presumed contaminants were pathogenic organisms would require a detailed validation approach to prove pathogenicity.
inflammatory changes could represent reactive immune responses to an earlier or nearby infection. Negative NGS studies in patients with neuroinflammatory diseases may also suggest the cause of such disorders is due to postinfectious or autoimmune inflammatory responses rather than an active infection.

Sequence-based results for patients in whom NGS studies were negative or inconclusive, as well as in cases supporting specific diagnoses, contained common skin bacteria and other contaminants that were not considered clinically relevant (appendix e-1). While we cannot rule out these species as pathogens, their presence in multiple samples collected from different patients widely spaced apart in time indicates that they are not likely to be causally related to the patients’ symptoms. For example, we observed the skin bacterium Propionibacterium acnes in almost all samples, illustrating the difficulty of capturing a pure sample without any microbial spillover from the intervening tissues. These findings illustrate the difficulty and complexity of identifying pathogens in human samples, which unavoidably contain some bacterial species because the human body is not a sterile environment and brain biopsies are obtained via skin incision, which is colonized with multiple bacteria. In addition, surgical procedures that include the use of irrigation solutions, biopsy handling, as well as processing methods (e.g., DNA extraction kits and other sequencing reagents) often contain bacterial DNA that may be captured by NGS methods especially when the concentration of microbial DNA is low.

An important limitation of computational analysis in microbiome studies is the size and completeness of genome sequence databases. Although thousands of bacterial and viral species have been sequenced, many others including human pathogens may yet be discovered, and unknown pathogens cannot be detected until their genomes are sequenced. Eukaryotic pathogens represent a larger challenge: many of these remain to be sequenced, and even those that have been sequenced are available only in draft form, with many gaps and errors in the publicly available genomes.

We described here the power of large-scale unbiased sequencing along with computational-based metagenomic analysis in the study of brain biopsies. Our study opens the possibility for more extensive use of NGS techniques in the identification of pathogens in patients with suspected CNS infections or neuroinflammatory disorders.

**AUTHOR CONTRIBUTIONS**

Steven L. Salzberg: study concept or design, computational biology data analysis, study supervision, drafting/revising the manuscript, obtaining funding. Florian P. Breitwieser: computational biology data analysis, revising manuscript. Anupama Kumar: tissue processing, clinical data analysis, revising manuscript. Haiping Hao: tissue processing, DNA/RNA sequencing. Peter Burger: neuropathology analysis, revising manuscript. Fausto J. Rodriguez: neuropathology analysis, revising manuscript. Michael Lim: neurosurgical procedure, revising manuscript. Alfredo Quinones-Hinojosa: neurosurgical procedure, revising manuscript. Gary L. Gallia: neurosurgical procedure, revising manuscript. Jeffrey A. Tornheim: clinical data analysis, revising manuscript. Michael T. Melia: clinical data analysis, revising manuscript. Cynthia L. Sears: clinical data analysis, revising manuscript. Carlos A. Pardo: study concept or design, neuropathology analysis, clinical data analysis, study supervision, drafting/revising the manuscript, obtaining funding.

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**DISCLOSURE**

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REFERENCES

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