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Retrospective Review of Clinical Utility of Shotgun Metagenomic Sequencing Testing of Cerebrospinal Fluid from a U.S. Tertiary Care Medical Center

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ABSTRACT Shotgun metagenomic sequencing can detect nucleic acids from bacteria, fungi, viruses, and/or parasites in clinical specimens; however, little data exist to guide its optimal application to clinical practice. We retrospectively reviewed results of shotgun metagenomic sequencing testing requested on cerebrospinal fluid samples submitted to an outside reference laboratory from December 2017 through December 2019. Of the 53 samples from Mayo Clinic patients, 47 were requested by neurologists, with infectious diseases consultation in 23 cases. The majority of patients presented with difficult-to-diagnose subacute or chronic conditions. Positive results were reported for 9 (17%) Mayo Clinic patient samples, with 6 interpreted as likely contamination. Potential pathogens reported included bunyavirus, human herpesvirus 7, and enterovirus D-68, ultimately impacting care in two cases. Twenty-seven additional samples were submitted from Mayo Clinic Laboratories reference clients, with positive results reported for three (11%): two with potential pathogens (West Nile virus and *Toxoplasma gondii*) and one with *Streptococcus* species with other bacteria below the reporting threshold (considered to represent contamination). Of 68 negative results, 10 included comments on decreased sensitivity due to high DNA background ($n = 5$), high RNA background ($n = 1$), insufficient RNA read depth ($n = 3$), or quality control (QC) failure with an external RNA control ($n = 1$). The overall positive-result rate was 15% (12/80), with 58% (7/12) of these interpreted as being inconsistent with the patient's clinical presentation. Overall, potential pathogens were found in a low percentage of cases, and positive results were often of unclear clinical significance. Testing was commonly employed in cases of diagnostic uncertainty and when immunotherapy was being considered.

KEYWORDS metagenomics, cerebrospinal fluid, next-generation sequencing

Determining the etiology of infectious neurological disorders and those that mimic infectious processes can be a diagnostic challenge. Despite an extensive toolbox for microbiological testing, 30 to 50% of patients with encephalitis are left without a definitive diagnosis (1–4). Shotgun metagenomic sequencing (SMS) is an agnostic approach to infectious diseases testing, allowing the simultaneous detection of genetic material from bacteria, viruses, fungi, and parasites. This approach is advantageous in that it does not rely on a provider's suspicion of a specific microbial pathogen(s), encompasses all pathogens in a single test, and can detect rare or novel pathogens for which routine, targeted diagnostic methods do not exist. For normally sterile clinical specimens such as cerebrospinal fluid (CSF), SMS would be a particularly useful diagnostic aid, as the absence of normal microbial flora in such specimens increases the likelihood that the detected organism(s) is involved in a pathogenic process.

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TABLE 1 Study population characteristics

Characteristic	Value
No. of subjects	80
Mayo Clinic	53
Mayo Clinic Laboratories	27
Mean age (yr) of subjects (range)	48 (11–85)
Mayo Clinic patients	49 (11–81)
Mayo Clinic Laboratories subjects	46 (11–85)
No. of female subjects (%)	38 (48)
Mayo Clinic patients	22 (42)
Mayo Clinic Laboratories subjects	16 (59)
No. of Mayo Clinic patients (%)	
Duration of symptoms	
Acute (≤3 wks)	9 (17)
Subacute (>3 wks but ≤3 mo)	17 (32)
Chronic (>3 mo)	27 (51)
Inpatient	25 (47)
Evaluated elsewhere without definitive diagnosis	33 (62)
Initiation or escalation of immunotherapy planned at time of ordering	27 (51)
Result altered treatment	2 (4)

To date, studies involving SMS of CSF have included proof-of-principle cases or case series alongside a single large prospective analysis of a tightly defined patient cohort (5–15). In this study, we performed a retrospective review assessing the real-world application of unrestricted SMS testing. The purpose of this study was to gather data to guide future diagnostic stewardship efforts by identifying patient or sample characteristics to maximize the diagnostic yield of SMS on CSF.

MATERIALS AND METHODS

Study design. All results on consecutive clinical CSF samples ($n = 80$) sent for SMS to an outside reference laboratory (https://nextgendiagnostics.ucsf.edu/providers/) (16) from December 2017 through December 2019 were reviewed. These samples originated from patients evaluated at Mayo Clinic (Rochester, MN), a 2,000-bed tertiary care medical center, or were submitted through Mayo Clinic Laboratories, an international reference laboratory. Demographic data, including age and gender, were collected for all samples. This study was approved by the Institutional Review Board of Mayo Clinic.

Chart review. For Mayo Clinic patients, chart review was performed by a clinical microbiologist (K. G. Rodino) and neurologist (M. Toledano) to extract the following data: (i) duration of illness at the time of presentation (acute illness defined as <3 weeks of symptoms, subacute defined as ≥3 weeks and <3 months, and chronic defined as ≥3 months), (ii) whether initiation or escalation of immunotherapy was being considered at the time of ordering, (iii) whether the patient was evaluated previously without a definitive diagnosis, (iv) the rationale for requesting SMS, and (v) results of routine CSF analysis (cell count, protein, and glucose) and infectious diseases tests (routine culture, nucleic acid amplification, and antigen/antibody testing). Finally, the impact of the SMS results on patient management and changes in treatment were assessed.

Data availability. All data used to review CSF SMS test results and to assess the impact on patient care are provided in the supplemental material. Sequence data are not available as clinical testing was performed at an outside reference laboratory.

RESULTS

Over a 2-year period, 80 CSF samples were submitted for SMS testing (53 from Mayo Clinic patients and 27 referred through Mayo Clinic Laboratories) (see Tables S1 and S2 in the supplemental material for details of clinical features and laboratory test results). The average ages of the subjects were similar for both groups, 49 years (range, 11 to 81 years) for Mayo Clinic patients and 46 years (range, 11 to 85 years) for Mayo Clinic Laboratories subjects, with an overall mean of 48 years (Table 1). Forty-two percent of the Mayo Clinic patients and 59% of the Mayo Clinic Laboratories subjects were female.

The majority of patients (44/53; 83%) evaluated at Mayo Clinic had subacute ($n = 17$) or chronic ($n = 27$) conditions, with 33 of 53 having received prior care elsewhere without a definitive diagnosis. Patients were equally split between inpatient ($n = 25$)

TABLE 2 Shotgun metagenomic sequencing results for cerebrospinal fluid specimens

Result	Total no. of subjects (%)	No. of Mayo Clinic patients (%)	No. of Mayo Clinic Laboratories subjects (%)
Positive	12 (15)	9 (17)	3 (11)
Potential pathogen	5 (6)	3 (6)	2 (7)
Likely contaminant	7 (9)	6 (11)	1 (4)
Negative	68 (85)	44 (83)	24 (89)

and outpatient ($n = 28$) settings, and 32% were immunocompromised at the time of presentation, including patients with prior solid-organ transplantation, malignancy, and/or immunological conditions. Encephalopathy was the most common presentation, with headache, amnesia, cognitive decline, and gait instability being the most common symptoms.

The majority of SMS testing (47/53) was requested by a neurologist, with infectious diseases, ophthalmology, and hospital internal medicine physicians being responsible for the remaining requests. An infectious diseases service was consulted or involved in care at the time of ordering in 23 of the 53 cases. Initiation or escalation of immunotherapy was under consideration in 27/53 cases, and the most common reason for requesting SMS was to rule out infection in complex cases (25/53) of unclear etiology. In four cases, SMS of CSF was pursued prior to performing brain biopsy.

Laboratory analysis of the CSF showed pleocytosis in 32/53 samples, with abnormal protein or glucose levels in 17/53 and 11/53, respectively. Of the 21 samples sent for SMS without evidence of pleocytosis, 6 were from patients with a prior sample showing pleocytosis. Only 6/53 patients had SMS requested on CSF obtained from their first documented lumbar puncture, with the majority having a prior lumbar puncture during outside Mayo Clinic evaluation or earlier in the course of care at Mayo Clinic. The routine infectious diseases testing performed varied for the sample that was sent for SMS testing. In general, extensive laboratory workup, including routine bacterial, fungal, and mycobacterial cultures; nucleic acid amplification testing; and serological testing, was performed on the same sample or an antecedent sample, covering a broad infectious differential.

Of the 80 samples sent for CSF SMS, 12 (15%) samples (9 from Mayo Clinic patients and 3 from Mayo Clinic Laboratories clients) were positive for at least one organism by SMS (Table 2). Five of the results represented potential pathogens, while the remaining 7 were most consistent with contamination. Among specimens from subjects cared for outside Mayo Clinic and submitted to Mayo Clinic Laboratories, probable pathogens detected included West Nile virus from a 74-year-old woman and *Toxoplasma gondii* from an 18-year-old man. *Streptococcus* species (thermophilic *Streptococcus* of dairy origin) was detected in the CSF of a 45-year-old man, but this result was believed to represent contamination given that this organism was codetected with *Lactobacillus* and *Morganella* species at levels below the reporting threshold value. Clinical information was not available for correlation in any of the Mayo Clinic Laboratories samples.

Three results from Mayo Clinic patients represented potential pathogens. Human herpesvirus 7 (HHV-7) was detected from a previously healthy 11-year-old boy who presented with fever, abdominal pain, urinary retention, and flaccid paraparesis. Magnetic resonance imaging (MRI) of the spine demonstrated longitudinally extensive gray matter-predominant T2 signal change without gadolinium enhancement. CSF studies revealed 26 nucleated cells with lymphocytic predominance but normal protein and glucose levels. Routine microbiological testing was notable for positive cytomegalovirus (CMV) IgM and IgG class antibodies in serum, while CMV PCR was negative from CSF. HHV-7 IgM and IgG class antibodies in serum were negative. The significance of the SMS result positive for HHV-7 was unclear, but given the negative CMV PCR result and incomplete recovery while receiving infusion therapy with intravenous immunoglobulins (IVIGs), oral valganciclovir therapy was initiated for 21 days; the patient improved. Bunyavirus was detected from a 59-year-old woman with a history of

follicular lymphoma presenting with chronic progressive cognitive decline and altered mental status. Brain MRI showed a symmetric T2 signal within the caudate, basal ganglia, and hippocampi. CSF collected for SMS showed only 1 nucleated cell with an elevated protein level (87 mg/dl) and a normal glucose level. The patient died prior to the return of the SMS result, and autopsy (including neuropathological examination) did not provide a definitive diagnosis, but the findings were thought to be most consistent with toxic-metabolic insult. Samples were sent to the CDC for further investigation; the results were still pending at the time of manuscript submission. The third positive SMS result thought to represent a potential pathogen was for enterovirus D (EV-D) (result most closely related to enterovirus D-68), which was detected in the CSF from a 29-year-old man with meningomyelitis presenting as acute gait unsteadiness and spastic quadriparesis. Brain MRI showed leptomeningeal and dural nodular enhancement most prominent in the basilar cisterns and upper cervical cord with associated hydrocephalus. CSF analysis showed 9 nucleated cells, 47% neutrophils, 41% lymphocytes, and 12% monocytes. In-house enterovirus PCR on the CSF as well as confirmatory testing at the Minnesota Department of Health laboratory on multiple sample types (including CSF) were negative. Despite the discrepancy between the patient's clinical and radiographic presentation and the reported EV-D result, IVIG infusion therapy was administered; there was no improvement.

The remaining 6 positive SMS results in Mayo Clinic patients were considered to represent contamination: *Bifidobacterium breve* (codetected with other gastrointestinal organisms below the reporting threshold), *Corynebacterium ureicelerivorans*, *Streptococcus* species (thermophilic *Streptococcus* of dairy origin codetected with *Lactobacillus* and *Morganella* species below the threshold), *Staphylococcus saprophyticus*, multiple bacterial genera consistent with oral microbiota, and multiple bacterial genera consistent with gastrointestinal microbiota. The cell count was normal in 4 CSF samples, while 7 and 50 nucleated cells were present in the remaining 2 samples with *C. ureicelerivorans* and *Streptococcus* species, respectively. Protein and glucose levels were slightly elevated in 2 of the samples, with negative routine infectious diseases testing. In all 6 cases, the patient care teams determined the SMS results to be unlikely to represent the causative pathogens, and treatment was not altered as other etiologies were pursued.

Of the 68 negative SMS results, 10 included comments about suboptimal assessment (e.g., "sample contains high DNA background; there is decreased sensitivity for detection of DNA viruses, bacteria, fungi, and parasites") due to a high DNA/RNA background, insufficient read depth, or failure of the external RNA control.

DISCUSSION

To our knowledge, this retrospective review of SMS results ($n = 80$) on clinical CSF samples tested over a 2-year period is the first to assess outcomes of unrestricted requests of such testing by patient care providers for both inpatients and outpatients at a tertiary medical care center and the second-largest study on the utility of CSF SMS testing. The goals of this study were to assess the patterns and diagnostic value of SMS testing in such a medical practice setting.

The patient population in which CSF SMS testing was pursued tended to have prolonged or chronic conditions for which they had sought prior medical evaluation without a definitive diagnosis or resolution. This observation may be driven, at least in part, by the role of the Mayo Clinic as a referral center. Although autoimmune and other noninfectious causes were more likely than infections in the differential diagnosis of most of these cases, a complete diagnostic evaluation for infectious causes was pursued, oftentimes prior to the initiation of immunotherapy. Such a evaluation was frequently prompted, as noted by Wilson et al., in cases of mild pleocytosis ($<$100 nucleated cells) where an autoimmune etiology may be most likely, but infection remains a consideration (15). The complexity of these cases and the risk of initiating immunotherapy in a patient with an infectious process were major reasons for requesting CSF SMS testing.

Twelve (15%) of 80 samples had positive CSF SMS results, with 58% of those likely representing clinical false-positive results. Of the six false-positive samples from Mayo Clinic patients, five had <10 nucleated cells and three had completely normal CSF analysis results. The pretest probability in testing normal CSF is typically low, increasing the likelihood of confounding SMS results. However, this finding is not generalizable and should be considered in the context of a patient's underlying immunosuppression. In our review, bunyavirus was detected from a severely immunocompromised patient with CSF showing only 1 nucleated cell.

Five (6%) CSF SMS results were thought to represent potential pathogens. *T. gondii* and West Nile virus presumably may have been able to be detected or confirmed through targeted nucleic acid amplification methods, but the lack of clinical history and other laboratory test results in these two Mayo Clinic Laboratories subjects limits our ability to verify this conclusion. In the Mayo Clinic patient cohort, three potential pathogens were detected (bunyavirus, HHV-7, and EV-D), representing 6% of the CSF SMS test results. As detailed in Results, the SMS results affected management in 2 (HHV-7 and EV-D) of the 3 patients since the SMS result positive for bunyavirus was reported postmortem.

Given the low return of potentially impactful results, cost is a consideration when ordering SMS testing. With only 5 potential pathogens detected in our study cohort, the cost per impactful result could be considered to be significant. While the cost to individual patients can be justified when the result impacts their well-being, the overall cost per actionable result can be high if employed in low-yield situations. Defining and identifying high-yield populations remain a challenge, however. Previous studies of SMS have indicated that the involvement of an infectious diseases specialist may help improve test utilization and result interpretation (17). In the Mayo Clinic cohort, an infectious diseases specialist was involved in 23 of 53 cases, with a potential pathogen being reported in 9% (2/23) of the cases where an infectious diseases specialist was involved, compared to 3% (1/30) in cases without infectious diseases involvement.

Rarely (6 of 53 cases) was SMS requested on CSF obtained from the first lumbar puncture, with the majority of patients having had extensive testing performed on a prior CSF sample, either in-house or at outside medical institutions. As CSF SMS is commonly used as a final diagnostic test, initial CSF samples may not be available for add-on testing by the time SMS is considered (18). Some pathogens (e.g., Powassan virus and Zika virus) may become rapidly undetectable from clinical CSF specimens as the disease progresses. Reserving a specimen for potential future SMS could be considered so long as this does not compromise routine testing. In cases with a high suspicion for an uncommon RNA virus, up-front ordering of SMS testing could be considered.

The interpretation of negative CSF SMS results was confounded in 10/68 (15%) cases that included reporting comments related to the potentially decreased sensitivity of the assay. In the Mayo Clinic patient cohort, repeat testing of CSF was not pursued in any of these cases, and this scenario seemed to be associated with the presence of high nucleated cell counts in the sample sent for SMS or in a recent antecedent sample. Decreased sensitivity of SMS testing due to high cell counts has been described previously (15).

In summary, CSF SMS detected a possible pathogen in 6% of cases and yielded clinical false-positive results in 9% of cases. No obvious clinical features or findings in routine CSF analysis correlated with CSF SMS results to help guide the request for such testing or increase the likelihood of an impactful result. Little diagnostic value was observed for testing CSF with normal cell counts, but exceptions can occur in immunocompromised individuals, as highlighted by a bunyavirus case. Patients' underlying levels of immunosuppression at the time of presentation and plans to initiate immunotherapy constituted two of the main considerations for requesting CSF SMS testing. CSF SMS is an adjunctive tool to detect rare pathogens in cases where the suspicion of infection is high and routine diagnostic microbiological methods fail to determine an infectious cause.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.02 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.2 MB.

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