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SUPPLEMENT ARTICLE

Metagenomic Next-Generation Sequencing for Diagnosis of Pediatric Meningitis and Encephalitis: A Review

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Metagenomic next-generation sequencing is a novel diagnostic test with the potential to revolutionize the diagnosis of pediatric meningitis and encephalitis through unbiased detection of bacteria, viruses, parasites, and fungi in cerebrospinal fluid. Current literature is mostly observational with variable indications, populations, and timing of testing with resulting variability in diagnostic yield and clinical impact. Diagnostic stewardship strategies are needed to direct testing toward high-impact pediatric populations, to optimize timing of testing, to ensure appropriate interpretation of results, and to guide prompt optimization of antimicrobials. This review highlights the high clinical potential of this test, though future studies are needed to gather clinical impact and cost-effectiveness data for specific indications in pediatric populations.

Key words. diagnostic stewardship; encephalitis; meningitis; metagenomic next-generation sequencing; pediatric.

ENCEPHALITIS IN PEDIATRICS

Around 700 US children per year are hospitalized with encephalitis at a cost of $64 000–260 000 per patient [1–3]. Forty percent will require intensive care, 3%–11% will die, and many will be left with persistent or permanent neurologic deficits, often requiring long-term rehabilitation [3, 4]. The incidence of meningitis in children is even higher, with a similar burden of disease, with 5%–10% mortality, and many patients with long-term neurologic sequelae [5, 6]. The major challenge in pediatric encephalitis and meningitis management is the difficult, and often delayed, etiologic diagnosis by clinicians.

As pediatric meningitis and encephalitis can be caused by a multitude of infectious agents, including viruses, bacteria, fungi, and parasites, and noninfectious etiologies, including primary neurologic, immune-mediated, neoplastic, metabolic, and toxicologic, several subspecialists are often involved with a wide spectrum of diagnostic tests performed [7]. Compounding this challenge, pediatric meningitis presents with common overlapping clinical features, such as fever and cerebrospinal fluid (CSF) pleocytosis, and encephalitis often with additional findings of encephalopathy, seizures, focal neurologic findings, and/or abnormal neuroimaging/electroencephalography, which are typically nonspecific to the underlying etiology. This leads to practice variation with wide variability in the diagnostic approach to these cases.

The traditional diagnostic approach to suspected central nervous system (CNS) infections in children has been low-yield, costly, and slow. Conventional microbiologic techniques rely primarily on culture, which requires the presence of viable organism in the CSF and takes time for growth to be detected on media, mainly limiting utility to detection of bacteria, fungi, and cultivable viruses. Serologic testing allows the detection of intrathecal production of pathogen-specific antibodies to organisms that not only may no longer be present in CSF at the time of clinical presentation but also may not yet be present in the acute setting.

Subsequently, molecular diagnostics have enabled more rapid and sensitive culture-independent diagnosis through the detection of pathogen nucleic acid by polymerase chain reaction (PCR). However, these tests still require clinician suspicion to direct pathogen-specific testing. Even with extensive clinician-directed pathogen-specific testing, a definitive etiologic diagnosis is identified in less than half of cases and often delayed, leading to prolonged hospitalization, delayed treatment initiation, and increased healthcare costs [2–4, 7, 8]. This diagnostic approach relies heavily on sufficient specimen volumes for multiple CSF tests. In pediatrics, lumbar punctures often require sedation and yield small specimen volumes, which may limit the quantity of testing performed or require repeat lumbar puncture and sedation, with the associated costs and risks, to collect more specimens. A prioritized approach to pathogen-specific testing is often necessary in pediatrics, but, given nonspecific clinical features, this can be inconsistent and variable between providers [4].

Advances in diagnostic technologies have created the potential for an evolution in the diagnostic approach to pediatric meningitis and encephalitis cases. The ability to multiplex

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several molecular targets on a single platform has enabled a syndromic diagnostic approach, allowing clinicians to test for the most common and clinically impactful pathogens with a single test using minimal specimen, transforming the approach to suspected CNS infections, particularly in pediatrics [9].

Most recently, unbiased sequencing emerged, including 16s ribosomal sequencing for bacteria-specific identification and metagenomic next-generation sequencing (mNGS). With increased utilization in the clinical arena, knowledge gaps regarding their optimal implementation and utilization for clinical use in pediatric meningitis and encephalitis have become apparent [10, 11]. In this review, we will focus on mNGS as a diagnostic tool for infectious causes of pediatric encephalitis and meningitis.

METAGENOMIC NEXT-GENERATION SEQUENCING

Metagenomic NGS is a novel unbiased sequencing approach that uses high-throughput technology to sequence billions of nucleic acid fragments simultaneously [12, 13]. Unlike traditional PCR methods that require specific primers, this offers a hypothesis-free sequencing method for pathogen identification [4]. Critically important bioinformatic analysis is needed to subtract host DNA in order to identify the microbial nucleic acids by matching DNA and RNA reads to genetic libraries of all known microorganisms, including bacteria, DNA and RNA viruses, fungi, and parasites [4, 13–15].

Due to its unbiased approach, mNGS has enormous potential to assist clinicians with the diagnostically challenging conundrum of pediatric meningitis and encephalitis. However, given the novelty of the assays, there are limited data on how best to implement mNGS for clinical practice. With more widespread use, the added difficulty of how to interpret mNGS test results has arisen as an additional challenge. A recent survey of 220 pediatric infectious disease providers of the Infectious Disease Society of America's Emerging Infections Network by Dehority et al. [10] found that 53% had used mNGS on CSF for diagnosis in children with meningitis or encephalitis but identified large variability in their knowledge and understanding of the use of mNGS. Many respondents were unsure of the best timing to send this test, with two-thirds reporting that they would only use it after standard testing excluded other infections, and half would send it only if the child were not improving [10]. Many providers reported challenges with the interpretation of negative results, with 68% noting that a negative result could not be used to effectively exclude infection [10]. There was also a general lack of consensus between pediatric neurology and infectious disease providers as far as subspecialty ownership of diagnosis and treatment of pediatric encephalitis patients, partly due to the variability in infectious and noninfectious causes [10]. This study highlighted current gaps in education and clinical guidelines for optimal implementation of this technology in the care of pediatric patients with meningitis and encephalitis [10].

Literature Search

We performed a systematic literature review on mNGS for the diagnosis of pediatric meningitis and encephalitis. The MEDLINE (via PubMed) database was searched using keywords "metagenomic sequencing pediatric encephalitis," "metagenomic sequencing pediatric meningitis," "metagenomic sequencing encephalitis," "NGS pediatric encephalitis," and "NGS pediatric meningitis," which produced 82 unique articles. Articles were screened for inclusion criteria, which included pediatric case reports, case series, case-control, and retrospective and prospective cohort studies regarding the use of mNGS in meningitis or encephalitis. Articles focusing on adult patients only, non-mNGS technologies, nonhuman specimens, and mNGS for applications other than meningitis or encephalitis were excluded. Only primary research studies were included. Validation-only studies without a clinical application were also excluded. A total of 29 articles met inclusion criteria (Figure 1). Two additional articles of the authors' own work meeting inclusion criteria were included that were not identified through the above search.

Case Reports

Eighteen case reports were found in the literature identifying pathogens found on mNGS of the CSF or brain tissue (Table 1). All of these reports were in patients presenting with meningitis, encephalitis, or both (meningoencephalitis), in which a single pathogen was detected and believed to be the potential causative agent [16–31]. At least, 8 (44%) of these reports resulted in an impact in clinical care with a change in antimicrobial therapy at the time of the mNGS result [18, 21, 24, 25, 27, 29, 30]. The remaining reports identified a pathogen either after the patient already recovered or expired or identified a viral pathogen without need for targeted therapy. These early case reports highlighted the exciting potential diagnostic role for mNGS in children with meningitis and encephalitis by demonstrating its ability to identify known causes of encephalitis, known pathogens not previously associated with encephalitis, and novel organisms.

Cohort and Case-Control Studies

A total of 13 studies were identified regarding the clinical use of mNGS in pediatric encephalitis and/or meningitis (Table 2). These included 8 retrospective cohorts, 3 prospective cohorts, and 2 case-control studies. There was wide variability in study populations with 5 of these studies also including adult patients. Although there are few prospective studies that have been conducted, current literature highlights key principles in the use of mNGS technology and establishes key knowledge gaps that need to be addressed.

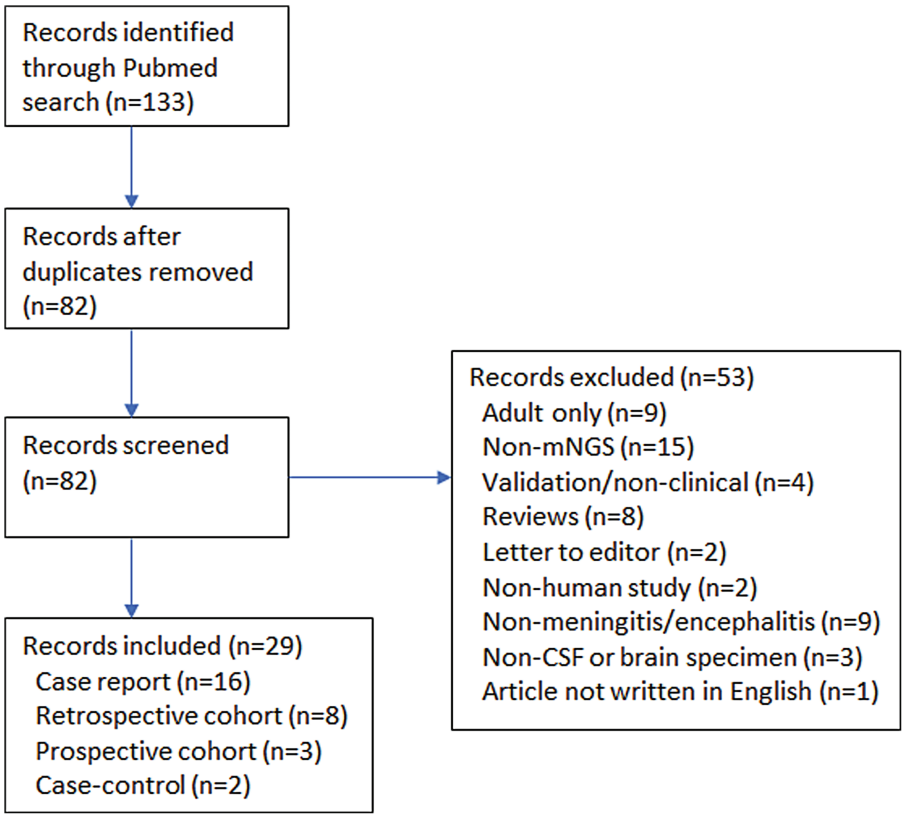

Figure 1. Methods of literature search using MEDLINE via PubMed.

Several studies that lacked a clear indication for testing had variable results regarding diagnostic yield and clinical applicability. Rodino et al. [42] described a cohort of patients with specimens sent to a reference laboratory, where testing was "unrestricted". The majority of these were referred patients presenting for reevaluation with subacute or chronic presentations, with only 25% of samples sent on the initial lumbar puncture. The most common indication for testing was to "rule out infection" [42]. They reported a positivity rate of 15%, with only 6% reflecting causative pathogens [42]. Likewise, Erdem et al. [36] performed mNGS on a cohort of pediatric patients with meningitis and/or encephalitis, of which 63% had a proven non-infectious cause or probable post-infectious syndrome. They concluded that mNGS identified a causative pathogen in only 1 patient (4%) [36]. However, only 37% of the case patients had either a proven viral cause or unknown etiology; they were only looking for viral pathogens and did not comment on other potential non-viral pathogens detected. In contrast, Xing et al. [41] took a different approach and only performed testing on those with definite or probable CNS infections, excluding patients with possible autoimmune encephalitis, which likely led to the reported increased diagnostic yield of 46%.

Some studies conversely used strict criterion for testing to validate the ability of mNGS to detect targeted pathogens from retrospective cohorts with known or suspected etiology. However, the lack of a consistent standard against which mNGS is compared hinders the currently available literature in this regard. For example, Zhang et al. [40] analyzed mNGS ability to detect *Streptococcus pneumoniae* in CSF specimens from patients with bacterial meningitis. They found a high sensitivity and specificity compared with conventional methods but demonstrated a minimal advantage over conventional methods [40]. However, the timing of specimens tested by mNGS was a major limitation, as these were more often collected later in the course of illness compared with those tested with conventional microbiologic testing [40]. Leon et al. [37] further evaluated the use of mNGS to identify cases of enterovirus A71 following a known outbreak. Metagenomic NGS increased detection rates of enterovirus A71 in the CSF compared with real-time PCR by 15% [37]. Wang et al. [43] performed mNGS on patients with confirmed or clinically suspected *Tuberculosis* (TB) meningitis. Metagenomic NGS increased the yield in clinically suspected cases by 26% overall but failed to detect TB in 17% of cases where conventional testing was positive [43]. These

Table 1. Pediatric Case Reports Identifying a Pathogen in Meningitis or Encephalitis Using mNGS

First Author/Year of Study	Patient Age	Clinical Presentation	Organism Identified	Specimen Source	Additional Testing	Clinical Outcome
Wilson 2014 [25]	14 y	Patient with SCID; meningoencephalitis with status epilepticus	*Leptospira santarosai*	CSF	Brain biopsy non-diagnostic; targeted PCR of CSF later confirmed diagnosis; serology negative	Initially suspected and treated for autoimmune disease with clinical worsening; later treated for leptospirosis with penicillin and recovered
Geng 2020 [18]	5 y	Previously healthy patient; prolonged fever and meningoencephalitis	*Brucella*	CSF	Brucella serology positive; later confirmed by positive blood culture	Treated for Brucellosis and improved
Mongkolrattanothai 2016 [21]	11 y	Previously healthy patient; meningoencephalitis	*Brucella*	CSF	Brucella IgM negative initially; later confirmatory serology positive	Initially treated for presumed TB meningitis; symptoms resolved 2 weeks after starting *Brucella* therapy with doxycycline and rifampin
Ortiz-Alcántara 2016 [23]	13 y	Previously healthy patient; meningitis	*Psychrobacter* sp.	CSF	Standard CSF cultures were negative	Patient died despite broad-spectrum antimicrobial therapy
Wang 2020 [24]	11 d	Full-term infant; meningitis and ventriculitis	*Ureaplasma parvum*	CSF	Standard CSF cultures were negative; confirmed by targeted *Ureaplasma* PCR	Improved on IV erythromycin for targeted *Ureaplasma* therapy
Greninger 2015 [19]	15 y	Patient with diabetes and celiac's disease; encephalitis	*Balamuthia mandrillaris*	CSF and brain tissue	Brain biopsy identified amebae; Targeted PCR of brain tissue later confirmed diagnosis	Initially suspected and treated for autoimmune disease with clinical worsening; patient died before treatment for *Balamuthia* initiated
Wu 2020 [29]	13 y	Previously healthy patient; cutaneous lesions and granulomatous encephalitis	*Balamuthia mandrillaris*	CSF	Targeted PCR later confirmed *B. mandrillaris* from skin biopsy	Initially treated for presumed TB with clinical deterioration; subsequently treated with amphotericin, 5-FU, and fluconazole with initial improvement but eventually died
Yang 2020 [31]	2 y	Previously healthy patient; prolonged fever and encephalitis	*Balamuthia mandrillaris*	CSF	TST was positive; TB PCR testing from CSF negative	Initially treated for TB, followed by IVIG and steroids with subsequent deterioration and patient died prior to mNGS result
Xie 2019 [30]	1 y	2 patients: previously healthy; meningoencephalitis	*Angiostrongylus cantonensis*	CSF	Patient 1: positive serum IgG for *A. cantonensis* Patient 2: negative CSF antibody for *A. cantonensis*	Both patients treated with albendazole and steroids with full recovery
Saporta-Keating 2018 [32]	11 y	Previously healthy patient; eosinophilic meningitis	*Taenia solium*	CSF	Confirmed by targeted PCR from CSF; positive IgG from CSF and serum	Treated with albendazole and steroids with full recovery
Edridge 2019 [17]	3 y	Previously healthy patient; encephalitis	Novel Orthobunyavirus (Ntwetwe virus)	CSF	Detected on both mNGS of CSF and plasma	Patient developed decorticate posturing and ultimately died prior to diagnosis
Phan 2016 [33]	6 y	Previously healthy patient; NMDA-receptor encephalitis	Novel densovirus (human CSF-associated densovirus 1)	CSF	Confirmed by positive targeted PCR in CSF; serum PCR negative	Patient treated for anti-NMDA receptor encephalitis with some improvement; unclear role of the novel virus
Cao 2020 [16]	17 y	Previously healthy patient; encephalitis and hemiparesis	Parvovirus B19	CSF	mNGS of plasma did not identify Parvovirus B19	Treated with IVIG and steroids empirically; eventually discharged home with partial deficits
Ikuta 2019 [20]	2 mo	Full-term infant; meningitis	Torque teno virus[a]	CSF	Standard cultures were negative	Patient recovered and discharged after 4 d of empiric antimicrobials and steroids
Wilson 2017 [26]	14 y	Renal transplant patient; meningoencephalitis	West Nile Virus	CSF	EBV PCR detected on CSF; WNV serologies from blood and CSF negative	Clinical worsening on antibiotics; treated for ADEM with IVIG, followed by ganciclovir for EBV; eventually discharged with persistent deficits

Table 1. Continued

First Author/Year of Study	Patient Age	Clinical Presentation	Organism Identified	Specimen Source	Additional Testing	Clinical Outcome
Olson 2019 [22]	13 mo	Previously health patient; gastroenteritis, hepatitis, and encephalopathy	HHV6	CSF	HHV6 also detected on multiplex PCR panel and quantitative PCR of CSF and serum	Improved with supportive care
Frémond 2015 [27]	14 y	Patient with XLA; progressive cognitive decline and seizures	Astrovirus	Brain tissue	Standard PCR for neurotropic viruses negative from CSF and brain biopsy	Treated with IVIG, steroids, and ribavirin; no further decline in functioning, improved seizures
Liu 2019 [28]	1 y	Previously healthy patient with encephalitis	HSV-1	CSF	No HSV PCR was sent; HSV IgG and IgM positive in serum; HSV IgG positive in CSF	Treated with acyclovir prior to mNGS result and patient recovered

Abbreviations: mNGS, metagenomic next-generation sequencing; SCID, severe combined immunodeficiency; PCR, polymerase chain reaction; CSF, cerebrospinal fluid; TB, tuberculosis; IV, intravenous; 5-FU, 5-fluorocytosine; IVIG, intravenous immunoglobulin; EBV, Epstein-Barr virus; WNV, West Nile virus; ADEM, acute demyelinating encephalomyelitis; HHV6, human herpes virus 6; XLA, X-lined agammaglobulinemia; HSV-1, herpes simplex virus 1 IgM, immunoglobulin M; NMDA, N-methyl D-aspartate; TST, tuberculin skin test.
[a]Although commonly considered a colonizing virus without clinical significance, the authors concluded that torque teno virus was the etiologic agent of meningitis in this patient.

studies illustrate the ability of mNGS to produce similar or slightly increased diagnostic yields to culture and targeted PCR, though using an unbiased assay in cases with a known etiology is not the intended use of the assay and is unlikely to add much clinical value.

The major advantage of mNGS is the ability to detect a multitude of infectious agents with a single assay without requiring a priori suspicion based on clinical features. Wilson et al. [4] demonstrated this best in their multicenter prospective study, in which they performed mNGS on 204 patients with meningitis and encephalitis in parallel with conventional testing. The median time to CSF sampling for mNGS was 3 days after initial presentation, demonstrating the real-life performance of this test [4]. They used broad criteria for inclusion, performing mNGS on patients with meningitis, encephalitis, or myelitis without an identified cause. Based on expert clinical review, 57 patients (28%) were ultimately determined to have an infectious etiology [4]. Metagenomic NGS discovered an infectious diagnosis in 13 patients (22%) that was not detected by conventional testing; 8 (62%) of these results affected clinical decision-making, as they were either not considered previously by the treating physicians or tested negative by conventional methods [4]. Further, in those with negative mNGS testing, clinicians noted that the mNGS results were helpful in providing reassurance to stop empiric therapy and expediting immunosuppressive therapies [4]. Overall, this study demonstrates the potential clinical impact of mNGS in patients with idiopathic meningitis and encephalitis when used at initial presentation, in conjunction with conventional testing.

Additional cohort studies have been performed highlighting similar results with mNGS used as an unbiased test. Haston et al. [34] performed a prospective cohort study to evaluate mNGS in pediatric patients with encephalitis of unknown etiology; however, they did not report results to clinicians. They identified 4 patients (20%) where mNGS would have made an earlier microbial diagnosis due to lack of availability of PCR-specific testing or rare pathogens [34]. Saha et al. [35] performed mNGS on banked CSF specimens in pediatric patients with idiopathic meningitis and found a potential causative pathogen in 40% of the cases. In particular, they identified 3 patients with Chikungunya virus neuroinvasive disease, which uncovered the etiologic agent of an unrecognized meningitis outbreak [35]. A study by Greninger et al. used mNGS in an attempt to identify pathogenic organisms in the CSF of children from an acute flaccid myelitis (AFM) cluster associated with an enterovirus D68 (EV-D68) outbreak. They did not detect EV-D68 or any other pathogens in the CSF, similar to conventional testing [38]. Metagenomic NGS strengthened the notion that there was no alternative agent responsible for the AFM outbreak. These studies further emphasize the strong clinical and public health potential of metagenomic sequencing to provide an unbiased investigation for etiology in challenging situations where

Table 2. Cohort and Case-Control Studies Using mNGS in Pediatric Meningitis and/or Encephalitis

First Author/Year of Study	Study Design	Patient Population	No. of Patients	Methodology	Outcome	Pathogens Detected	Conclusions
Haston 2020 [34]	Prospective cohort	Pediatric immunocompetent patients hospitalized with encephalitis of unknown etiology	20	mNGS performed for investigational use only on CSF specimens	mNGS identified pathogen(s) in 6 patients, of which 4 were thought to be causative; higher diagnostic yield in patients with CSF abnormalities	·Presumed pathogens: *Mycoplasma bovis*, parvovirus B19, *Neisseria meningiditis*, *Balamuthia mandrillaris* ·Presumed non-pathogens: *Cladophialophora* sp., tobacco mosaic virus, human bocavirus	mNGS can be used as adjunctive therapy in pediatric patients with encephalitis of unknown etiology with higher yield in those with CSF abnormalities
Saha 2019 [35]	Case-control	Pediatric cases of idiopathic meningitis compared with known infectious and noninfectious causes of meningitis	25 cases; 36 positive controls; 30 negative controls	mNGS performed on saved specimens of cases, positive and negative controls as part of a larger validation study	mNGS identified a potential causative pathogen in 40% of the cases; 3 were identified as Chikungunya virus identifying an unrecognized meningitis outbreak; mNGS correctly identified pathogens in 69% of positive controls; no pathogens were detected in negative controls	· Bacteria: *Salmonella enterica*, *Stenotrophomonas maltophilia*, *Bacillus cereus*, *Mycobacterium tuberculosis* ·Viruses: Chikungunya virus, mumps virus, enterovirus B	mNGS can be used to complement conventional diagnostic testing for clinical diagnostics; it also has a potential role for epidemiologic surveillance and outbreak investigations
Erdem 2020 [36]	Case-control	Pediatric patients hospitalized with encephalitis or meningoencephalitis (cases): 15% proven viral, 22% probable post-infectious, 41% proven noninfectious, 22% no etiology identified	27 cases; 10 controls	mNGS was performed on cases and compared with positive controls (known enteroviral meningitis) and negative controls (primary intracranial hypertension)	mNGS identified 1 pathogenic agent among cases; nonpathogenic agent in 13 patients; correctly identified enterovirus in all positive controls; detected no pathogens in negative controls	·Case patients: 1 West Nile virus and 12 torque Teno virus ·Positive controls: 4 enterovirus and 1 torque Teno virus	mNGS did not offer a diagnostic advantage to conventional testing
Leon 2020 [37]	Retrospective cohort	Pediatric patients with brainstem encephalitis or meningoencephalitis during an outbreak of Enterovirus A71	20	mNGS performed on CSF of patients with brainstem encephalitis or meningitis/encephalitis compared with results of qRT-PCR	mNGS enhanced Enterovirus detection in CSF from 0 with qRT-PCR to 3 with mNGS (15%)	Enterovirus A71	mNGS increases detection of Enterovirus A71 in CSF
Greninger 2015 [38]	Retrospective cohort	Pediatric cases of confirmed AFM (43% positive for EV-D68 by conventional oropharyngeal or nasopharyngeal PCR)	14	mNGS performed on saved CSF specimens from patients with clinical diagnosis of AFM	No pathogens were detected from CSF samples from the EV-D68 positive or negative patients	None	mNGS was used to further strengthen the notion that there is no alternative agent responsible for EV-D68 AFM; mNGS detection is limited by its ability to detect pathogens that directly invade the CNS
Kawada 2016 [39]	Retrospective cohort	Pediatric patients with acute encephalitis/encephalopathy of unknown etiology	16	mNGS performed on saved CSF of patients to evaluate potential pathogens	3 samples were positive for viral pathogens	Coxsackievirus A9 and mumps virus	mNGS has potential to identify viral pathogens implicated in encephalitis not otherwise identified from conventional testing
Hasan 2020 [12]	Retrospective cohort	Pediatric patients with CSF sent to the microbiology laboratory for clinical testing due to suspected CNS infection; 26% with positive CSF pathogen by culture or PCR, excluding RNA viruses	74	A clinical validation study of an in-house mNGS platform performed on saved CSF specimens to determine diagnostic accuracy compared with conventional testing	21 samples were positive by mNGS (28%); 100% sensitivity, 95% specificity, and 96% accuracy compared with conventional methods; 3 additional pathogens were detected that were not detected by conventional methods	Pathogens by mNGS only: *Streptococcus agalactiae*, *Streptococcus parasanguinous*, HSV2	A clinical mNGS approach has similar diagnostic accuracy compared with conventional methods with faster turnaround time but offered little advantage over conventional testing in this study

Table 2. Continued

First Author/Year of Study	Study Design	Patient Population	No. of Patients	Methodology	Outcome	Pathogens Detected	Conclusions
Zhang 2019 [40]	Retrospective cohort	Pediatric patients with proven or probable bacterial meningitis	135	mNGS performed on saved CSF specimens to evaluate for *S. pneumo* and compared with the results of conventional testing	37 (27%) identified as *S. pneumo* by conventional testing (culture or antigen testing); 32 (24%) were positive by mNGS with 6 positive only via mNGS	*Streptococcus pneumoniae*	mNGS shows high sensitivity and specificity when compared with combined culture/antigen testing for *S. pneumo* meningitis
Wilson 2019 [4]	Multicenter, prospective cohort	Adult and pediatric (23%) patients with meningitis and encephalitis; 41% immunocompromised; 49% required intensive care	204	mNGS performed clinically on CSF samples in parallel with conventional testing	NGS identified additional 13 infections (22%), of which 8 affected clinical decision-making; 26 patients had negative mNGS where microbial diagnosis was made through conventional testing	Pathogens by mNGS only: ·Viruses: SLEV, hepatitis E virus, EBV, echovirus, MW polyomavirus ·Bacteria: *Streptococcus agalactiae, Neisseria meningitidis, Nocardia farcinica, Candida tropicalis, Klebsiella aerogenes, Streptococcus mitis, Enterococcus faecalis*	mNGS improves infectious diagnosis of meningitis and encephalitis and has a clinical impact in some cases; clinical interpretation from experts in the "tumor-board" approach can strengthen the clinical use of mNGS
Xing 2020 [41]	Multicenter, prospective cohort	Adults and pediatric patients with meningitis and/or encephalitis with high clinical suspicion for infectious cause; excluded those with autoimmune encephalitis	213	mNGS performed on CSF specimens after conventional methods; only included patients with definite or probable CNS infections based on clinical review	Positive detection rate of definite CNS infections was 57.0% and 41% for probable infections	·Viruses: HSV, VZV, EBV, CMV, adenovirus ·Bacteria: *Streptococcus* sp., *Klebsiella, Listeria, Nocardia, Brucella, Stenotrophomonas, H. flu, E. coli, Aggregatibacter, Neisseria* ·Fungi: *Aspergillus, Cryptococcus* sp.	mNGS effectively identifies infectious causes of CNS disease and should be used in conjunction with conventional testing
Rodino 2020 [42]	Retrospective cohort	Pediatric and adult patients; cases of diagnostic uncertainty	80	mNGS performed on CSF specimens sent to reference laboratory for clinical sampling	Positive results in 12 patients (15%); only 5 thought to be causative pathogens (6%) with 2 impacting clinical care	Causative pathogens: bunyavirus, HHV7, EV-D68, West Nile virus, *Toxoplasma gondii*	Low overall positivity rate with positive results often of unclear clinical significance
Wang 2019 [43]	Retrospective cohort	Adults and pediatric (13%) patients with definite or clinically suspected TB meningitis	23	mNGS was performed retrospectively on CSF samples in comparison to conventional methods from the first lumbar puncture	mNGS confirmed TB in 18 patients (78%) vs conventional testing in 12 patients (52%); combination of mNGS and conventional methods increased detection rate to 96%	*Mycobacterium Tuberculosis* complex	mNGS outperformed conventional methods in the diagnosis of Tb meningitis and should be used in conjunction with conventional methods to increase diagnostic yield
Carbo 2020 [44]	Retrospective cohort	Adult and pediatric (41%) hematologic patients with encephalitis of unknown etiology	41	A clinical validation study using viral metagenomics with enriched viral capture probes to detect viral pathogens on saved CSF and brain biopsy specimens negative by conventional methods	mNGS detected an additional 5 viral pathogens (12%) not detected by conventional PCR methods	BK polyomavirus, Hepatitis E virus, HHV6, EBV	Hematologic patients with encephalitis may benefit from early use of viral metagenomics to enhance diagnosis

Abbreviations: CSF, cerebrospinal fluid; CNS, central nervous system; HSV2, herpes simplex virus 2; SLEV, St. Louis encephalitis virus; EBV, Epstein-Barr virus; CMV, cytomegalovirus; VZV, varicella zoster virus; *E. coli*, *Escherichia coli*; *H. flu*, *Haemophilus influenzae*; HHV6, human herpes virus 6; HHV7, human herpes virus 7; EV-D68, enterovirus D68; AFM, acute flaccid myelitis; PCR, polymerase chain reaction; mNGS, metagenomic next-generation sequencing; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction.

Table 3. Summary of Takeaways on the Use of mNGS for Diagnosis of Suspected Central Nervous System Infections in Children

• **mNGS can augment, but should not replace, conventional microbiologic testing** *It is most useful in cases with diagnostic uncertainty where syndromic testing is unable to identify an etiology and directing pathogen-specific targeted testing is challenging due to lack of clinically differentiating features.*
• **Consideration of timing is key to clinical impact and cost-effectiveness of mNGS** *This includes both when to send CSF for testing in the diagnostic work-up/clinical course and turnaround time to result in order to impact clinical decision-making.*
• **mNGS can only detect infections with pathogenic nucleic acid in CSF** *Serology, tissue sampling, and testing of nonsterile sites for shedding of associated pathogens can augment diagnostic work-up for suspected CNS infections.*
• **mNGS results must be interpreted carefully in the clinical context of the patient scenario** *The unbiased and sensitive nature of mNGS may detect unsuspected pathogens which may be clinically relevant or unassociated based on compatibility with the clinical presentation.*

Abbreviation: mNGS, metagenomic next-generation sequencing.

infectious causes of meningitis and encephalitis outbreaks or cases are suspected.

These studies additionally highlight the potential increased diagnostic yield of mNGS as an adjunctive, but not stand-alone, test. Studies with the highest clinical impact used mNGS in parallel with conventional culture, PCR, and serology. In Wilson et al. [4], mNGS failed to detect an organism in CSF in several cases where conventional methods made the diagnosis outside the CSF. The majority of these were due to lack of the organism or pathogen nucleic acid in CSF; the diagnosis was made either by serology, on testing of brain tissue, or at an alternative site [4]. For TB meningitis specifically, Wang et al. [43] demonstrated that the combination of mNGS and conventional testing increased the diagnostic yield in suspected cases to 96%. Saha et al. [35] performed a case-control study with idiopathic meningitis cases and known infectious cases as positive controls. Although mNGS offered an advantage by identifying a potential agent in 40% of their idiopathic cases, it missed 31% of pathogens in their known infectious cases [35]. These studies strengthen the principle that metagenomic sequencing is best used to complement conventional diagnostic testing, optimizing clinical impact when used in parallel.

It is important to note that many of these studies used mNGS sequentially, only after conventional diagnostics have failed to identify an etiology, which is likely suboptimal timing. The majority of these studies were conducted retrospectively on saved CSF specimens and often not from the first lumbar puncture. Zhang et al. [40] specifically report that the CSF specimens used for mNGS were collected later than those obtained for conventional testing, often several days into their course of illness, and after initiation of antibiotic therapy. In Wilson et al. [4], although the majority of cases were sampled from first lumbar puncture, 35% were sampled upon second or later lumbar puncture, often after receiving antimicrobial therapy, thus potentially decreasing the diagnostic yield. Future studies should address optimal timing for the use of mNGS, to determine its clinical applicability at presentation or initial CSF sampling.

These early studies offer evidence for the strong clinical potential for mNGS to improve the diagnostic yield in infectious cases of pediatric meningitis and encephalitis. However, there is a lack of robust data to guide clinicians on the optimal implementation, use, and interpretation of this novel diagnostic test. Rodino et al. [42] determined that with unrestricted mNGS testing, the majority (58%) of their positive results were of unclear clinical significance and would not impact clinical care, highlighting a lack of clear clinical significance as a major limitation to its use. Erdem et al. [36] identified 13 cases of torque teno virus, which is a commonly identified ubiquitous virus and was believed to be clinically insignificant in their patients, further stressing the need for clinical interpretation. As there is currently no standard for interpretation, Wilson et al. [4] used an innovative "tumor board"-type approach, where a panel of clinicians with expertise in CNS infections discussed the results in the context of the clinical setting to determine their clinical significance specific to each patient. The results of the sequencing board allowed for better consensus regarding the decision to stop empiric therapy, rule out co-infections, diagnose infectious syndromes, and expedite treatment for noninfectious causes [4]. In addition, this board discussed results of supplementary mNGS analyses, including viral genotyping and antimicrobial resistance, tracking of new or rare pathogens, detection of pathogens below the reporting threshold, and more accurate species identification [4]. One particular dilemma that occurs with mNGS results is whether to report pathogens detected at low levels not meeting the threshold cutoff. The ability of the laboratory to discuss the results with the treating clinicians in this "tumor board"-type approach can facilitate consensus on whether the organism is considered causative or incidental. As mNGS may detect nonpathogenic, unsuspected, or novel organisms, this approach may be useful to guide proper interpretation and clinical decision-making, highlighting the need for diagnostic stewardship and interpretive diagnostic microbiology with this and other emerging technologies. Summary of takeaways in Table 3.

Limitations of mNGS

There are several important limitations to mNGS despite its revolutionary diagnostic potential. These include access, turnaround time, cost, and limitations of CSF testing for CNS infections. Despite increasing knowledge of this technology,

access to metagenomic sequencing is still a barrier. Most clinical microbiology laboratories lack clinical capabilities to perform in-house clinical mNGS testing and, therefore, samples have to be sent to specific reference laboratories. This creates a longer turnaround time, which is a major limitation to rapid diagnosis. Despite markedly improved turnaround times, the median time for laboratory processing is still around 3-4 days; with added transit time to reference laboratories, total turnaround times can be longer than 10 days [4, 45]. Prolonged turnaround times dilute clinical impact, as critical management decisions surrounding empiric and targeted therapies often occur in the acute phase immediately following presentation. To bring this assay to the clinical laboratory, the estimated cost is around $100 000 in supplies for development, validation, and bioinformatics expertise alone [46, 47]. Though pre-sample costs of mNGS testing have come down significantly, each assay costs between $1000 and 2500 per sample analyzed and remains considerably more expensive than conventional testing [45, 48]. Until it is more widely available, this will continue to be a limitation for most centers.

In addition, samples are still limited by the presence of extra host DNA typically in specimens with high nucleated cell counts >200 cells per cubic milliliter [4, 42]. This may limit the ability of mNGS in pediatric meningitis in particular, where diagnosis is critical, but significantly elevated nucleated cell counts are common, often in the range of thousands. Further, CSF with high red blood cells due to traumatic taps is exceedingly common in pediatrics, further decreasing the sensitivity of the assay and proportion of mNGS controls that may fail.

Despite being the most inclusive test for CSF, there are still many infectious organisms that are not actively present in CSF at the time of clinical presentation, limiting the use of any test that detects for the presence of an organism at the time of sample collection. Notable examples include EV-D68, West Nile virus, California encephalitis virus, and other neuroinvasive arboviruses [4, 34, 36, 38]. Accordingly, mNGS should be paired with serologic testing to detect host response and maximize yield [4]. Platforms to conduct pan-viral serologic testing of CSF, using phage-based or microarray chip-based platforms, are used in the research setting but not yet available clinically. These platforms demonstrated increased diagnostic yield in enterovirus D68 AFM, where CSF and nonsterile site testing was negative for viral nucleic acid by PCR or mNGS, but enterovirus antibodies, and no other consistent viral antibodies, were present in CSF [49, 50]. Pan-viral serologic testing may become the host-response complement to unbiased mNGS, and, when paired, these 2 complementary approaches may provide a more comprehensive diagnostic evaluation in pediatric meningitis and encephalitis.

CONCLUSIONS

Metagenomic NGS has the potential to positively impact clinical care as an unbiased sequencing test for the diagnosis of infectious causes of pediatric meningitis and encephalitis. The primary advantage of mNGS is the ability to detect the most known bacteria, viruses, fungi, and parasites in CSF without requiring clinical suspicion to direct pathogen-specific testing, but it remains most useful when conducted in parallel with conventional testing and when sent early in the clinical course. Diagnostic stewardship strategies are essential to guide the implementation of this test in clinical practice and optimize impact. Future diagnostic stewardship studies are needed to provide more robust data to better define high-yield patient populations and indications, optimize timing of testing, and evaluate real-time decision support strategies, such as the use of a "tumor board"-type approach, to guide interpretation and management decisions. Further, as turnaround time and high cost remain major limitations, prospective cost-effectiveness studies are needed to determine the value added by mNGS testing. With the advent of mNGS, the diagnostic research pipeline has added another powerful instrument to our clinical toolkit to tackle the age-old challenge of identifying the etiology of pediatric meningitis and encephalitis; it is now up to us as pediatric infectious disease clinicians and researchers to learn how best to use it.

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