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LEADING ARTICLE

Clinical Metagenomic Next-Generation Sequencing for Diagnosis of Central Nervous System Infections: Advances and Challenges

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Abstract

Central nervous system (CNS) infections carry a substantial burden of morbidity and mortality worldwide, and accurate and timely diagnosis is required to optimize management. Metagenomic next-generation sequencing (mNGS) has proven to be a valuable tool in detecting pathogens in patients with suspected CNS infection. By sequencing microbial nucleic acids present in a patient's cerebrospinal fluid, brain tissue, or samples collected outside of the CNS, such as plasma, mNGS can detect a wide range of pathogens, including rare, unexpected, and/or fastidious organisms. Furthermore, its target-agnostic approach allows for the identification of both known and novel pathogens. This is particularly useful in cases where conventional diagnostic methods fail to provide an answer. In addition, mNGS can detect multiple microorganisms simultaneously, which is crucial in cases of mixed infections without a clear predominant pathogen. Overall, clinical mNGS testing can help expedite the diagnostic process for CNS infections, guide appropriate management decisions, and ultimately improve clinical outcomes. However, there are key challenges surrounding its use that need to be considered to fully leverage its clinical impact. For example, only a few specialized laboratories offer clinical mNGS due to the complexity of both the laboratory methods and analysis pipelines. Clinicians interpreting mNGS results must be aware of both false negatives—as mNGS is a direct detection modality and requires a sufficient amount of microbial nucleic acid to be present in the sample tested—and false positives—as mNGS detects environmental microbes and their nucleic acids, despite best practices to minimize contamination. Additionally, current costs and turnaround times limit broader implementation of clinical mNGS. Finally, there is uncertainty regarding the best practices for clinical utilization of mNGS, and further work is needed to define the optimal patient population(s), syndrome(s), and time of testing to implement clinical mNGS.

Key Points

Metagenomic next-generation sequencing (mNGS) can detect a wide range of organisms, including rare and unexpected pathogens.

mNGS is increasingly used to diagnose central nervous system infection.

Clinicians should be aware of the strengths and limitations of diagnostic mNGS for individual patients, while ongoing and future work will help guide its optimal use at a population level.

1 Introduction

Diagnosing central nervous system (CNS) infections continues to pose major challenges. The rate of CNS infection in high-income countries is 11/100,000, with a fatality rate ranging from 5.5 to 21.1%[1]. The clinical presentation is frequently non-specific, and the etiological agents can be diverse and include viruses, bacteria, fungi, and parasites [2]. Immunocompromised patients, who represent an increasing proportion of the population, present additional diagnostic challenges given their increased susceptibility to unusual pathogens, atypical clinical presentations, and blunted antibody responses [3–6]. In addition, there are many mimics to infectious encephalitis, including post-infectious encephalitis, autoimmune encephalitis, and malignancy, which can occur at a similar frequency to infection and further complicate diagnosis [7–9]. A substantial proportion of patients with suspected CNS infection have an unknown etiology

Extended author information available on the last page of the article

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(13% to upward of 50% for pediatric patients in some settings) or incorrect diagnosis [10–14]. Failure to consider potential etiologies on the differential diagnosis and order appropriate testing may account for more than 75% of missed diagnoses [13]. While multiplex polymerase chain reaction (PCR) assays (e.g., BioFire FilmArray Meningitis/Encephalitis panel) are increasingly used to diagnose CNS infections, they target a limited number of pathogens, limiting the utility for nosocomial infections or immunocompromised patients [2]. Metagenomic sequencing, also referred to as metagenomic next-generation sequencing (mNGS), is a powerful, target-agnostic approach that can simultaneously sequence most nucleic acids in a sample and taxonomically classify the microbes present. As mNGS becomes more prevalent in clinical practice, users must be well informed about both its capabilities and limitations.

2 Metagenomic Sequencing Technology is Rapidly Advancing

Next-generation sequencing (NGS) consists of massively parallel sequencing of billions of molecules of nucleic acids, allowing for rapid, scalable and high-resolution genomic characterization. Ongoing advances in NGS technology have allowed for lower operating costs with a more user-friendly interface, enabling its broader adoption for testing in clinical settings, including microbiology [15]. The two most common applications of NGS in microbiology laboratories are targeted NGS and whole-genome sequencing (WGS). Targeted NGS focuses on specific genomic regions of interest and utilizes target enrichment, for example, by PCR, before sequencing to enhance analytical sensitivity. Examples of this approach include bacterial 16S ribosomal sequencing and fungal internal transcribed spacer (ITS) sequencing [16, 17]. Targeted NGS has proven particularly valuable for identifying complex polymicrobial infections, strain typing, and rapid genotypic prediction of antimicrobial resistance [18–20]. Whole-genome sequencing (WGS), which generates an entire pathogen genome sequence, is commonly used for surveillance and outbreak investigation [21]. WGS has also been used to identify bacterial and fungal species in the clinical setting [22, 23]. Despite targeted NGS allowing culture-free, highly sensitive pathogen detection with genetic information, it is still limited by the need to specify targets of interest [24].

Metagenomic NGS (mNGS) is a method that utilizes the expansive scale of NGS to sequence nucleic acids (DNA and/or RNA) directly from a clinical specimen. After nucleic acid is extracted, it undergoes "library construction," in which RNA is first converted to complementary DNA (cDNA), most often using random primers, and the cDNA or DNA is amplified, indexed, and has adapters added, all without the use of pathogen-specific primers or probes. The resulting sequencing data are then submitted to a computational pipeline that aligns and taxonomically classifies each read to microbial reference sequences found in public and/or internally curated databases, including complete and partial genomes, and thus allow for identification of the presence or absence of a pathogen. The relatively unbiased nature of mNGS allows for hypothesis-free detection of a wide range of microorganisms, which is especially valuable for unusual, fastidious, and emerging pathogens [3] (Fig. 1).

Currently, multiple NGS sequencing platforms are available, and each brings different advantages when applied to mNGS [25]. The widely used short-read sequencing platforms provided by Illumina and Beijing Genomics Institute (BGI) generate short reads (up to 300 bases) through bridge amplification, sequencing by synthesis, and fluorescent-labeled reversible terminators. An advantage of these platforms for mNGS is their extremely low error rate. Nanopore sequencing using instruments provided by Oxford Nanopore Technologies (ONT) can generate longer reads (up to tens or even hundreds of thousands of reads) using nanopore chemistry, in which single-stranded nucleic acid passes through a protein nanopore, causing electrical current disruptions that are specific to each nucleotide. An advantage of ONT for mNGS is the opportunity to provide real-time base calling, allowing analysis to be performed before the sequencing run is complete. There are continual advances in throughput and cost for all of these platforms. Currently, each platform offers a range of machines with different levels of output, from a few million reads to tens of billions of reads per run, allowing flexibility in scale and very high throughput when needed.

3 Metagenomic Analysis is Complex

Metagenomic analysis of clinical samples yields a large amount of data, with output files typically containing 5–25 million short reads. These sequence data are analyzed through automated bioinformatics pipelines using curated reference databases to perform quality filtering, subtraction of host reads, classification of reads by taxonomy, and alignment of microbial reads to reference genome sequences. The identified microbes must be reviewed, and relevant findings summarized into a meaningful clinical report [26, 27].

Even for CSF samples with low cell counts, the vast majority of sequence reads are of human origin, with only small fractions of a percent belonging to microbial species [28]. Samples with higher cellular content have proportionally more human nucleic acid, making it difficult to achieve high sensitivity for pathogen detection and accurate quantitation [29]. Methods to deplete human or enrich microbial reads can be incorporated into mNGS

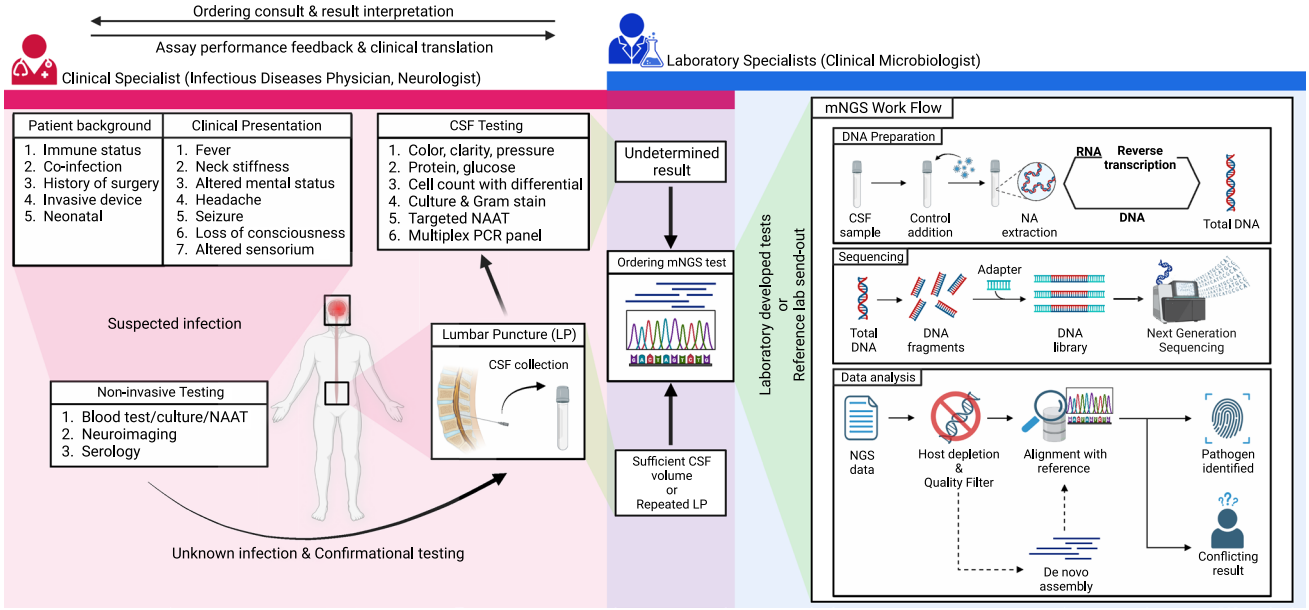

Fig. 1 Overview of the metagenomic next-generation sequencing (mNGS) workflow. This figure illustrates the step-by-step utilization of mNGS for central nervous system diagnosis, from the bedside to the interpretation of final results. It underscores the importance of collaborative effort between clinicians and laboratory specialists in achieving a consensus on diagnostic decisions. NAAT, nucleic acid amplification test; CSF, cerebrospinal fluid; NA, nucleic acid

workflows, such as differential cellular lysis, nuclease digestion, depletion of human nucleic acid, and enrichment of microbial nucleic acid using primer sets or probe capture [30]. Recent studies also suggest that long-read technology can improve the specificity of taxonomic assignment and allow for comparable sensitivity for diagnosis of meningitis cases at lower read depths [31].

The quality of library preparation, sequence generation, analysis methods, and reference databases are all critical to ensure reliable results from mNGS testing. Different approaches to library generation can introduce bias, and sequencing errors can lead to organism misidentification and inaccurate taxonomic classification [32]. Databases must be evaluated for comprehensiveness and accuracy, especially since most publicly available datasets contain misannotations, inaccurate assemblies, and contaminating sequences in assembled organism genomes [33]. A particularly notable example is a series of studies showing that components of the human Y chromosome were misannotated as bacterial genomes, which could have led investigators to inadvertently draw erroneous conclusions about the microbiome of males versus females [34, 35]. Bioinformatic tools for data analysis vary in performance and require performance assessment when used for clinical diagnosis to prevent misidentification during human subtraction, microbial alignment, and taxonomic classification [36–38]. It is largely because of these complexities in data analysis that diagnostic mNGS is limited to a small number of laboratories with the necessary bioinformatic expertise.

4 Metagenomic Sequencing can be Useful for Clinical Diagnostics and More

Clinically validated mNGS testing and analysis have been available to the North American commercial market since 2016. The University of California, San Francisco (UCSF) has been at the forefront of mNGS diagnosis for CNS infections and offers clinically validated mNGS tests for CSF specimens [39]. The Karius test (Karius Inc., California, USA) is a commercially available, validated mNGS test for the detection of microbial cell-free DNA (cfDNA) from plasma [40]. Plasma mNGS provides a non-invasive testing alternative for diagnosis of CNS infection that may be especially valuable in certain cases; however, there are currently limited data supporting this use at present [41–44].

Numerous case studies and case series have demonstrated the value of mNGS in patients with suspected CNS infection. The foundational example was the case of a critically ill child with meningoencephalitis from *Leptospira santarosai* that was missed by conventional testing but detected by mNGS, leading to a favorable clinical outcome [45]. In addition to bacteria, mNGS can detect mycobacteria, fungi, protozoa, and ameba [46–49]. It has been widely used to diagnose viral CNS infections, including those from unexpected

and even novel viruses [50, 51]. mNGS can be particularly useful in immunocompromised patients who may have atypical clinical presentations, such as chronic arboviral infection, and may have blunted antibody responses, rendering conventional testing negative [52]. For bacterial CNS infections, mNGS can improve diagnosis in patients receiving antimicrobial therapy prior to CSF sampling, when culture yield is low [53]. In this patient population, mNGS has been found to have higher sensitivity than culture: 52.5% versus 34.2% in one study, and 23.7% versus 19.3% in another [54, 55].

An important benefit of mNGS is that it not only identifies microbes, but also provides detailed genetic information about them. For example, mNGS can be used to predict antimicrobial resistance; in a patient with post-neurosurgical *Acinetobacter baumannii* CNS infection, mNGS was able to detect a carbapenem resistance gene and a possible fluoroquinolone resistance mutation [56]. In addition, mNGS offers the opportunity to investigate infectious disease epidemiology through pathogen genome sequence assembly and phylogenetic analysis. For example, CSF mNGS was used in Bangladesh to identify an outbreak of neuroinvasive Chikungunya virus caused by a single viral strain and was able to diagnose 40% of cases of idiopathic meningitis cases [57]. Thus, mNGS can facilitate surveillance for known, novel, and reemerging pathogens. Improvements in data sharing and analysis tools could allow for real-time molecular epidemiology of community-acquired meningitis, especially when routine diagnostic methods fail to achieve a diagnosis [58]. Finally, in addition to microbial sequence data, mNGS also generates host transcriptomic data, which may aid in diagnosis and further our understanding of pathogenesis. In one study, host transcriptomic analysis from mNGS data identified more than 400 differentially expressed genes that clustered by infectious diagnosis, including viral and bacterial meningitis [59]. Thus, mNGS holds tremendous appeal as both a diagnostic tool and as a bridge to public health and scientific investigation.

5 There is Growing Data Regarding the Utility and Limitations of mNGS in CNS Infection

Case reports and case series such as those described above have generated considerable enthusiasm for diagnostic mNGS; however, the impact and role of this approach cannot be defined by positive case reports alone. The field has certainly been subject to positive publication bias, and caution is needed to avoid well-intentioned but misinformed use. More recent studies have focused on understanding the performance characteristics of mNGS when applied more broadly.

A key example is a study of 204 pediatric and adult patients in the USA, in which CNS infection was diagnosed in 57 patients (28%) using both conventional methods and mNGS [60]. Among those 57 patients, results from mNGS and conventional methods were concordant in 19 (33%). The diagnosis was made by mNGS alone in 13 patients (22%), and mNGS missed infections in 26 patients (45%), including 11 diagnosed by serologic testing only, 7 diagnosed from tissue samples other than CSF, and 8 with low pathogen titers in CSF.

These results illustrate some of the important limitations of mNGS and underscore the nuances in comparing results from mNGS with conventional testing, which encompasses a range of methodologies and specimen types. As further illustration of the importance of comparator type, among 44 patients in another study with CNS infection diagnosed by conventional testing, mNGS was positive in 83% of those diagnosed by CSF PCR but only 36% of those diagnosed by serology or other testing [61]. In the same study, mNGS identified a pathogen in 3 of 24 patients (12%) with no diagnosis achieved by conventional testing [61]. The performance of mNGS also varies by pathogen type. For example, in a study of 213 patients, DNA-only mNGS had a sensitivity of 73% for bacterial meningitis, 77% for cryptococcal meningitis, and 80% for cerebral aspergillosis, but only 27% for tuberculous meningitis [62].

Finally, it is important to determine whether there are specific patient populations in which diagnostic mNGS has higher yield. As described above, immunocompromised patients may be particularly likely to benefit from mNGS testing. In a study of 88 people with advanced human immunodeficiency virus (HIV) infection and confirmed or suspected CNS infection, mNGS identified a plausible pathogen in 50 (57%) but missed pathogens in 18 (20%), most frequently tuberculous meningitis [63]. In the same study, mNGS led to modification of treatment in 21 (24%) patients and increased confidence in continuation of original therapy in 30 (34%) patients [63].

Pediatric patients may also benefit from mNGS, as they typically undergo lower volume CSF collection from lumbar puncture, limiting the amount of sample available for conventional testing. In a systematic review of 26 pediatric studies, comprising 15 case reports and 11 case series, mNGS was performed on CSF samples from 529 pediatric patients, and potential causal pathogens were identified in 22.1% [64]. In one specific study of 101 neonatal intensive care unit (NICU) patients, mNGS had a diagnostic yield of 20%, compared with just 5% for conventional methods [65]. Thus, there is substantial need for large-scale implementation studies to evaluate the performance of mNGS sequencing for specific patient populations and syndromes.

6 Interpreting the Results from mNGS can be Difficult

Despite the remarkable advances in diagnostic mNGS, especially for CNS infection, there are important limitations and challenges that should be considered by clinicians when requesting mNGS and interpreting its results. Although mNGS can detect a broader range of organisms compared with other molecular methods, its limit of detection can be inferior to other well-optimized molecular targeted assays (leading to lower sensitivity), and it may detect clinically irrelevant microbes that are present in the environment, assay reagents, and patient sample, leading to lower specificity [39, 61].

It is also crucial to understand that a negative mNGS result does not rule out infection. When interpreting negative results, clinicians must consider the technical limitations at both wet bench and dry bench (bioinformatic) levels. First and foremost, mNGS, like pathogen-specific PCR, is a direct detection modality, and the assay is only able to identify a pathogen if microbial nucleic acid is present in the tested sample at the time of collection. Some infections, notably arthropod-borne viruses (arboviruses) such as West Nile virus, often do not have detectable RNA in CSF by the time the patient presents for care [61, 66]. Other factors that can decrease the sensitivity of mNGS include a low level of pathogen nucleic acid relative to host nucleic acid in the sample, the need for highly efficient nucleic acid extraction, the stability of nucleic acid through the pre-analytical stage, and the sequencing depth targeted during sequencing, as deeper sequencing may be more likely to detect low-abundance pathogens, albeit at higher costs. To address these sensitivity limitations, multiple laboratory methods have been employed to increase the "signal-to-noise" ratio, including enrichment of pathogen nucleic acid using hybridization probes or primers [61, 67, 68]. Though helpful, these methods can also add time and expense, and also increase the risk of reagent and/or laboratory contamination. Another approach is to deplete host nucleic acid, which is often achieved by differential lysis methods (selective lysis of human cells followed by extracellular DNA degradation from DNases). Host depletion methods show various levels of effectiveness, depending on the sample type and biomass [61, 69]. More recently, CRISPR-based methods have shown promise for efficient human and bacterial ribosomal RNA depletion, but can be expensive and time consuming [70, 71]. Further efforts to improve the signal-to-noise ratio while maintaining speed and low cost will greatly enhance the utility of diagnostic mNGS.

At the dry bench, the main technical limitations to the sensitivity of mNGS are related to the bioinformatic pipelines and databases used. For example, if an organism does not have adequate sequence representation in the database, it will not be detected by mNGS. Potential pathogens that share little or no nucleotide sequence similarity to reference genomes in existing databases are not routinely identified using mNGS as existing methods of pathogen discovery, such as translated nucleotide alignment to pathogen databases, de novo assembly, analysis of dinucleotide composition, and/or aligners for distantly related sequences such as PSI-BLAST, are computationally expensive [72, 73]. Furthermore, the clinical significance and pathogenic potential of any newly discovered pathogen, or whether it is even a human-infecting virus, is nearly always unclear at the time of discovery, requiring extensive confirmatory follow-up investigation [74].

Finding an unexpected positive result from mNGS testing presents a different challenge. Clinicians should be aware that mNGS can detect microbes present in the environment, including the patient's skin, the laboratory environment, and the reagents used, despite careful adherence to sterile practices. For example, in mNGS clinical validation studies, 7% of samples had multiple bacterial genera present, which were interpreted as environmental contaminants and skin flora [39]. Additionally, reads from anaerobic bacteria have been commonly found in samples from healthy individuals, a phenomenon described as "DNAemia" [75]. Distinguishing environmental microbes from those truly present in the sample can be partly addressed through the robust use of negative controls during the pre-analytical step and sequencing process, but cannot mitigate exogenous microbial nucleic acid introduced during sample collection and transportation.

Furthermore, mNGS can detect microbes that are truly present in the sample but are unlikely to be causing disease. Some microbes are of unclear clinical significance, such as human herpesvirus 6 (HHV-6) and Epstein–Barr virus (EBV) [61, 67, 76]. For example, in a single-center study, Epstein–Barr virus (EBV) and BK polyomavirus was detected in 9/276 and 1/276 patients, respectively, while the clinical presentation did not match these findings. Detection of DNA from these viruses may only represent detection of integrated DNA or episomes [77]. Overall, if a positive mNGS result is not clearly aligned with the clinical presentation, additional testing using secondary or orthogonal methods may be required; this in turn can be challenging if there is not an orthogonal method available, or there is not sufficient volume of CSF remaining for additional testing.

Given the complexities of mNGS, collaborative engagement between clinical specialists (neurology, infectious diseases) and laboratory specialists (clinical microbiologists) is critical when deciding to request mNGS, selecting sample(s) to analyze, and interpreting results [78]. The analytical limitations of mNGS are better navigated by laboratory specialists, while clinical specialists can best contextualize the

results for an individual patient. A collaborative diagnostic team is essential for the generation of actionable results with mNGS testing [79–81]. Ultimately, collaborative care serves the dual purpose of benefiting patient outcomes and advancing the standards of care of the medical institution (Fig. 1).

7 There are Challenges in Broad Implementation of Diagnostic Metagenomic Sequencing

We are at a critical time in the field of diagnostic mNGS, since it is an available test that is increasingly being used, while many of the key questions surrounding widespread implementation have not yet been answered. While mNGS is currently used under specific circumstances, and often as a test of "last resort," it may be more broadly implemented in the future by addressing key challenges (Fig. 2). One important challenge in mNGS diagnostic testing is translating the results (whether positive or negative) into meaningful impact within a clinically relevant timeframe. Turnaround time (TAT) is a key factor in diagnosing CNS infections, as delayed antimicrobial therapy can lead to poor clinical outcomes, especially with bacterial meningitis and herpes simplex virus (HSV) encephalitis [82–86]. While mNGS assays can be performed in the laboratory within 24–48 h, [15, 87] current "real world" TAT is often 1–3 weeks after sample collection [88]. This is because of delays in the decision to send a sample for mNGS after it has been collected, as well as the need for batching and quality control within the performing laboratory. This is within a comparable range to bacterial, mycobacterial, and fungal cultures, where the TAT ranges from several hours to several weeks [85, 89]. In contrast, the TATs for in-house serology and PCR assays typically range from 2 h to 8 h [85]. Thus, there is room for improvement in mNGS TAT to optimize its usefulness. Although not yet implemented as a clinical test, research studies have demonstrated a 6 h mNGS assay using Oxford Nanopore technology [41, 90].

Another important challenge in diagnostic mNGS is its cost. When diagnostic mNGS assays were launched in 2016–2017, the per-sample cost was around $2,000; this has increased with inflation to $2,500–3,500 in 2024 [91–93]. However, mNGS may yet prove to be cost-effective by leading to fewer overall tests, avoidance of invasive procedures, antibiotic de-escalation, and shortened length of hospital stay [3]. In addition, advanced laboratory methods, such as removal of host nucleic acid, may help with cost reduction, as it reduces the sequence throughput needed for detection of pathogens with adequate sensitivity. The other key to cutting costs is automation, as labor is increasingly expensive, and labor shortages are expected to be a long-lasting challenge for clinical laboratories [94]. The ideal clinical mNGS test would be fully automated and implemented on high-throughput platforms, similar to those used for singleplex or multiplex PCR testing.

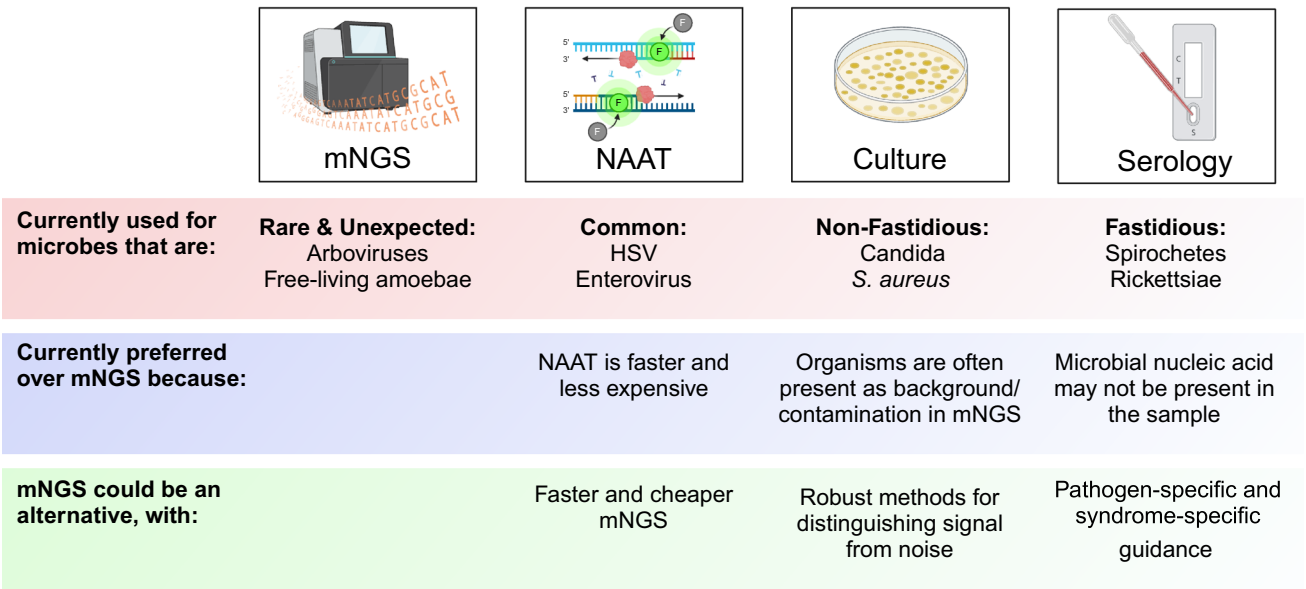

Fig. 2 Current and potential future approaches to pathogen detection. Whereas mNGS is currently employed to identify rare and unexpected pathogens, it also offers the opportunity to detect pathogens that are currently identified through nucleic acid amplification tests (NAAT), culture, and serology, with the following considerations: to be considered an alternative to NAAT, mNGS needs to be more rapid and cost-effective; to be considered an alternative to culture, mNGS needs to be more able to clearly distinguish true-positives from background/contamination; to be considered an alternative to serology, further investigation is needed to determine whether nucleic acid is truly present in a pathogen-specific, sample-specific, and likely patient-specific manner

In addition to practical challenges such as TAT, ease of use, and cost, mNGS faces complex regulatory challenges. Although the Food and Drug Administration (FDA) in the past has published a draft guideline (now rescinded), there has not been an FDA-approved mNGS assay for in vitro diagnostics [95]. Thus, diagnostic laboratories must decide whether it is in the best interest of their patients and other stakeholders to develop a laboratory-developed test (LDT) following CLIA regulations. LDT development for mNGS is a complex process due to the lack of professional guidelines, regulatory guidance, and standardized material. Additionally, the required instruments and bioinformatics expertise are generally only available in large academic medical centers. Therefore, most institutions currently send specimens to reference laboratories that run mNGS LDTs. Analytical and clinical performance data are available for the most widely used mNGS assays [39, 40]. However, assays performed with different reagents, sequencing platforms, and bioinformatics pipelines are considered as different tests, as each may exhibit its own unique analytical and clinical performance. The LDT approach faces additional challenges since the FDA in September of 2023 proposed a rule to enforce its regulation on LDTs, which will require reviews and approval processes similar to other in vitro diagnostic (IVD) products [96]. As of writing this manuscript, more than 2100 public comments have been submitted, expressing concerns about the burdens for laboratories to follow this rule, including reduced access to patient testing and obstacles to innovation [97].

Finally, a central challenge in broadly implementing diagnostic mNGS is uncertainty regarding best practices for test utilization. Because of the tremendous potential of diagnostic mNGS, its capabilities may be overestimated and lead to clinical expectations that exceed realistic possibilities. In part, this may be due to positive publication bias, as compelling diagnoses made by mNGS are often published, overshadowing studies with negative results and minimizing the diagnostic saga that can continue after mNGS. This can be somewhat mitigated through case-by-case collaboration between clinicians and clinical microbiologists, as described above. In addition, more information is needed to inform policies for diagnostic stewardship on a broader scale. Diagnostic stewardship revolves around five core principles: ordering the best test, for the best patient population, in the most appropriate clinical syndrome, at the right time in the disease course, with the optimal specimen. While the best specimen type for meningitis and encephalitis mNGS testing is usually CSF, key questions remain unknown regarding the other core principles. Should test utilization focus on immunocompromised, critically ill, or patients with specific risk factors? When is the best time to utilize mNGS: early in the clinical presentation or later, after conventional diagnostics remain unrevealing? How do pre-analytic steps such as host depletion, sequencing depth, and platform impact raw data, which are then processed differentially through various bioinformatics pipelines and databases? These questions require further investigation, including data collection on mNGS usage, clinical impact, and diagnostic stewardship studies with standardized outcomes.

8 Future Directions

As diagnostic mNGS becomes increasingly accessible to clinicians evaluating patients with suspected CNS infection, it is important to ensure that providers are knowledgeable about its strengths and limitations and how to interpret results. This can be achieved through educational efforts (e.g., seminars, papers, and other specific instructional content) and by the ongoing development of infectious disease and medical microbiology subspecialists with expertise in diagnostic mNGS. In addition, it is important in the field of diagnostic mNGS to define the optimal conditions under which to use this tool.

Key questions include:

- Which patient populations or clinical syndromes benefit most from mNGS testing?
- When in a diagnostic workup should mNGS be used?
- As an increasing number of diagnostic mNGS services become available, do their laboratory and analysis workflows meet standards for quality and reproducibility?
- Are results being interpreted within a diagnostic framework that is shared across the community of experts in CNS infection and diagnostic mNGS?

The first two of these questions underscore the need for refined implementation studies to define the optimal use cases for mNGS. Prior investigations have shown a range of utility, with pathogens identified by mNGS in 12–34% of individuals with suspected CNS infection and negative conventional testing results [60, 61, 67, 98]. They suggest that mNGS can change clinical management in 4–50% of patients who undergo testing [99–101]. To some clinicians and clinical microbiologists, these numbers are too low and too variable to support mainstream use of mNGS. An important caveat, however, is that mNGS is most often used as a test of last resort after extensive negative clinical testing; its utility may be better demonstrated early in infection, leveraging the capacity to capture both common and rare diagnoses. These questions should be addressed by multicenter studies with standardized patient populations and test timing, along with routine results interpretation by diagnostic mNGS subspecialists.

Identifying the patient populations for whom mNGS testing will have the diagnostic yield as well as the optimal time

of testing would, in turn, inform the development of formal clinical guidance, which will help address the last two questions above. Guidance for mNGS will need to include recommendations for test indications, sample collection including type and timing, and results interpretation. The first step toward formal guidance for mNGS occurred with the 2023 revised Modified Duke Criteria for Infective Endocarditis, which includes mNGS as an acceptable method for satisfying the pathologic criteria [102].

Although it is likely that the utility of mNGS will continue to increase with improved methods, decreased cost, and optimized implementation, it will never be a "silver bullet" for infectious disease diagnostics. Its results will always need to be carefully interpreted by clinical teams with a solid understanding of mNGS methodology, limitations, and strengths. Its usage should also be guided and monitored by a multidisciplinary diagnostic stewardship team consisting of both clinical and laboratory specialists to maximize cost effectiveness and clinical utility.

Declarations

Ethics Approval Not applicable.

Consent (Participation & Publication) Not applicable.

Data Availability Statement Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Code Availability Not applicable.

Financial Disclosures C.Y.C. is a founder of Delve Bio and on the scientific advisory board for Delve Bio, Flightpath Biosciences, Biomeme, Mammoth Biosciences, BiomeSense, and Poppy Health. He is also an inventor on US patent 11380421, "Pathogen detection using next generation sequencing," under which algorithms for taxonomic classification, filtering and pathogen detection are used by SURPI+ software. C.Y.C. receives research support from Delve Bio and Abbott Laboratories, Inc. on projects related to metagenomic next-generation sequencing for diagnosis of infections. D.G. receives research support from Illumina, IDbyDNA, and T2 biosciences, and also serves as a consultant for bioMerieux, QuidelOrtho, and Diasorin. C.A.H. receives research support from Ridagene, Seegene, and Promega, and also serves as a consultant for Inflammatix. S.M. is employed by Delve Bio and holds multiple patents related to metagenomic sequencing for infectious disease. K.T.T. serves as a consultant for Delve Bio.

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Author Contributions L.D.S., C.A.H., and A.P. conceptualized the manuscript and designed the figures. All authors wrote the first draft and edited drafts the manuscript. A.P. edited the final manuscript.

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