Advances in molecular diagnostic testing for central nervous system infections

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Purpose of review
Central nervous system (CNS) infections present an ongoing diagnostic challenge for clinicians, with an aetiological agent remaining unidentified in the majority of cases even in high-income settings. This review summarizes developments in a range of diagnostic methods published in the past 18 months.

Recent findings
Several commercial assays exist for the detection of viral, bacterial and fungal pathogens using single multiplex PCR. Multicentre validation of the Biofire FilmArray panel illustrated high sensitivity for bacterial and fungal pathogens, but poor results for *Cryptococcus* species detection. The development of microarray cards for bacterial CNS pathogens shows promise but requires further validation. Few developments have been made in proteomics and transcriptomics, contrasted with significant increase in the use of metagenomic (or unbiased) sequencing. Novel viruses causing CNS infection have been described using this technique but contamination, cost, expertise and turnaround time requirements remain restrictive. Finally, the development of Gene Xpert and Ultra has revolutionized tuberculosis meningitis diagnostics with newly released recommendations for their use from the WHO.

Summary
Progress has been made in the clinical validation and international recommendation of PCR-based tests for CNS infections. Sequencing techniques present the most dynamic field, although significant ongoing challenges persist.

Keywords
central nervous system infections, molecular diagnostics, PCR, sequencing

INTRODUCTION

Current data demonstrate that even in the best-resourced centres, up to two-thirds of cases of central nervous system (CNS) infections remain undiagnosed [1,2]. Successful patient outcomes are clearly associated with diagnostic confirmation, and we urgently need to improve the accuracy and accessibility of tools at our disposal. Further, the diagnosis of CNS infections is associated with more complex challenges owing to the difficulties of direct sampling of the site of disease via lumbar puncture or brain biopsy, and the extensive list of potential causes.

This review summarizes recent advances in the field of molecular diagnostics for CNS infections. We provide a critical appraisal of the literature over the last 18 months, and highlight the most relevant developments.

PCR AND NUCLEIC ACID DETECTION METHODS

Because of high sensitivity and specificity, PCR has been the gold-standard diagnostic method for viral CNS infections for almost two decades. As well as a turnaround time of only 1–2 h for real-time PCR results, the quantification of viral nucleic acid allows an evaluation of response to treatment and an of prognosis [3,4]. PCR can now be used for the diagnosis of bacterial and fungal CNS infection, allowing for the combination of several targets in a single PCR reaction (multiplex PCR), which has revolutionized diagnostic facilities. Multiple commercial assays are now available (e.g. SeeGene and Fast Track Diagnostics). One of these, the Biofire FilmArray panel, requires minimal hands-on time

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KEY POINTS

- Up to two-thirds of suspected CNS infections remain undiagnosed, even in high-income settings.
- Molecular assays can now be used for the simultaneous detection of multiple CNS pathogens (bacterial, fungal and viral), with variable sensitivity.
- The Xpert MTB/RIF Ultra assay is an automated, cartridge-based system for the molecular diagnosis of TB meningitis that has demonstrated a high sensitivity (95% compared to culture) in HIV-infected individuals and is recommended by WHO for this purpose.
- Metagenomic or unbiased sequencing of CSF in cases of meningitis or encephalitis has facilitated the identification of novel viruses, and unexpected bacterial, viral and fungal causative pathogens, as well as potentially nonpathogenic viruses. Progress is being made in reducing the number of gaps including cost and skills required for laboratory as well as bioinformatics analysis, the management of contaminants and turnaround time.

and incorporates automated nucleic acid extraction, reverse transcription, amplification and results in a single machine in under 1 h. A recent multicentre evaluation compared the Biofire CNS infection panel against routine testing (culture or individual real-time PCR). The Biofire had a sensitivity of 98% (78/80) for bacterial pathogens, 90% (145/161) for viral pathogens and 52% (26/50) for the single fungal pathogen on the panel, *Cryptococcus neoformans/gattii* [5]. The disappointing performance of the assay for fungal detection highlights the importance of utilizing additional simple and low-cost nonmolecular diagnostics, such as cryptococcal antigen, in combination with PCR.

A TaqMan array card has been developed to detect 21 pathogens (13 viruses, 6 bacteria and 2 parasites) with claims of increased sensitivity by utilizing a microfluidic card, which performs 384 simultaneous PCR reactions; each sample loading port being connected to 48 reaction wells. A study evaluating the TaqMan array card demonstrated good results in an initial evaluation of a selected number of samples. However, in a retrospective study of consecutive patient CSF samples with lower pathogen concentrations, the assay showed poor sensitivity compared to routine testing [6].

Rapid detection of pathogen DNA can be achieved through microarray assays where species-specific oligonucleotide probes are fixed to wells, and pathogens are detected by fluorescent dye hybridization. Microarrays for the detection of multiple CNS pathogens have been developed including one which targets seven bacteria known to cause acute bacterial meningitis. When tested against 24 purulent CSF samples which had high clinical suspicion for bacterial meningitis, the microarray detected a pathogen in 21 samples (88%). Further, in seven in which the bacterial culture was negative, the microarray detected a pathogen consistent with the clinical picture [7]. This positive finding required further optimization and testing, ideally with reduced cost of the single-use plate.

Rapid multiplex molecular diagnostic tests for CNS infection, such as those described above, have been shown to reduce hospitalization and duration of antibiotic treatment [8], strengthening the argument for further development. The challenge, however, remains the high cost of single test cardriges (approximately 200 USD per test for Biofire), equipment requirements and the limited range of targeted pathogens. Immunocompromised patients are susceptible to a range of less common organisms, and nosocomial meningitis or infections related to shunts and other intracranial devices are often polymicrobial. The range of targets required for multiplex PCR, microarray or Taqman array cards is therefore beyond what is reasonable for these patients and novel methods are required.

SEQUENCING

Sequencing technologies can be divided into two broad categories depending on the platform used; short-read sequencing performed most commonly on the Illumina (Hi-Seq and Mi-Seq) or Ion Torrent platforms (Ion Torrent S5 or S5 XL), or long-read sequencing performed most commonly on Oxford Nanopore's MinION. Short-read sequencing is most often used for metagenomic sequencing (also called unbiased or shot-gun sequencing), and target-amplicon sequencing [9]. Metagenomic sequencing involves the generation of a DNA strand or complimentary DNA strand from each fragment of genomic material in a sample followed with bioinformatic analysis to first remove human sequences and assembly remaining fragments with comparison to publically available sequence databases (Fig. 1) [10,11▪▪]. Novel pathogens can be identified by partial matches to the conserved regions of known sequences. In the case of metagenomics sequencing, high accuracy is required, which is provided by Illumina sequencers that report 0.1–1% error rates per nucleotide base. Target-amplicon or target-enrichment is used when a specific pathogen or group of pathogens is suspected. For example, identification of bacterial and fungal DNA can involve PCR amplification of the conserved gene coding for the ribosomal subunits 16 s (bacteria) and 18 s (fungal), among others, followed by

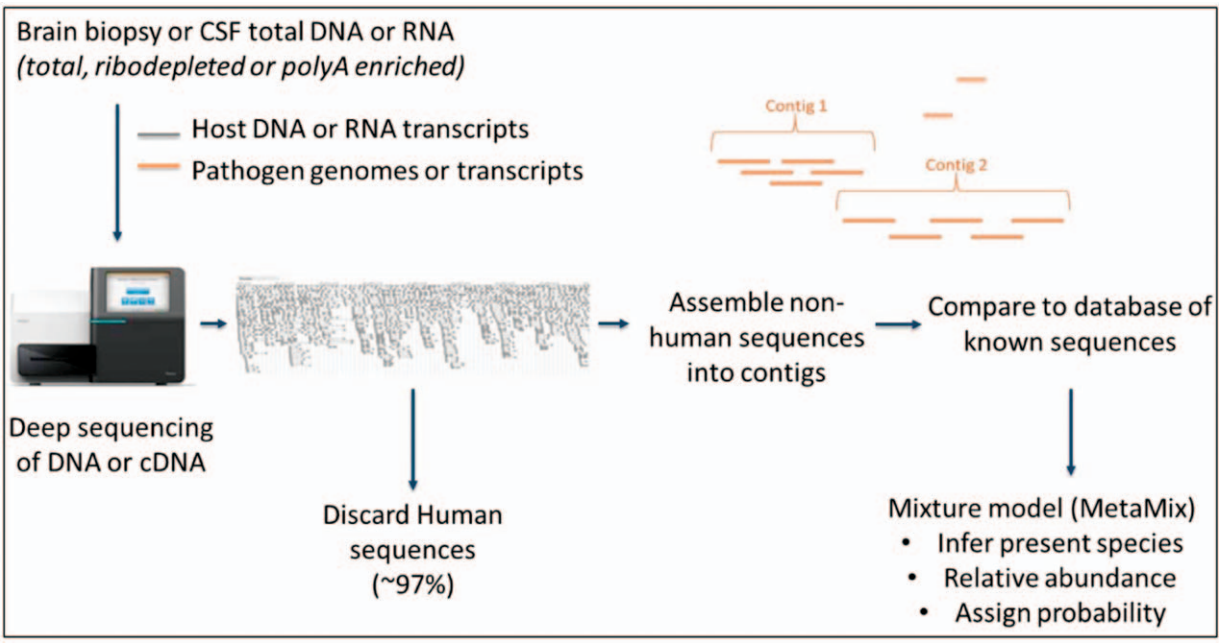

FIGURE 1. Illustration of typical metagenomics workflow (MetaMix is an example of an analysis tool (reference [10] open access)) [reprinted from reference [11▪▪]].

sequencing of the product for species identifications [12▪▪]. This technique is now in common use in high-income settings for the detection of bacteria when cultures are negative. The recent publication of metagenomics sequencing analysis of samples from patients with brain abscess and bacterial meningitis highlights some advantages over culture; bacteria have been identified when standard cultures were negative, and multiple plausible bacteria were identified when culture revealed a single organism [13–15]. Sequencing, of course, does not identify phenotypical antibacterial sensitivity and therefore offers only supplementary or complementary analysis in bacterial CNS infection.

Long-read sequencing utilizes nanopore technology, which allows the identification of pathogens by reading sequence data from RNA or DNA molecules as they transit through a pore 10^{-9} m in size. This technology continues to improve and several devices now exist from the Oxford Nanopore group; MinION, GridION, PromethION and SmidgION, the latter being the size of a USB and designed to work with a smartphone. Although the turnaround time for samples is shorter than with short-read sequence platforms (~4 h from sample processing to result interpretation), the error rate on reading genomic data remains higher (up to 10% error rate), limiting its use for cases where a novel pathogen is suspected.

A systematic review of studies, which have used metagenomics for the diagnosis of patients with test-negative encephalitis, identified 44 case reports [11▪▪]. In these 44 reports, 18 novel, 5 rare and 5 unexpected pathogens (bacterial, viral and fungal) were identified, highlighting the advantage of this technique over the targeted molecular methods mentioned earlier. A further review was published in January 2019, which expanded the criteria to include meningitis and meningo-encephalitis but limited this to viral pathogens [16▪▪]. In this review, studies that used metagenomic approaches identified potential viruses causing disease in 41 patients, 10 of which were unexpected (Fig. 2) [16▪▪]. A lack of consistency in laboratory (platform, protocols) and bioinformatics analysis (pipelines, databases), and the lack of standardized negative controls samples are described in the review as 'striking'. This heterogeneity is expected when a new technology emerges but we agree that the studies illustrate the significant challenges of replicability and potentially validity of the findings; an area of particular concern when applied to human health. Further publication of previously undescribed viruses isolated from CSF has since emerged [17], in keeping with an exponential trend for the identification of novel pathogens using metagenomics [18▪]. Further discussion of the limitations of the emerging trend for novel or unexpected pathogens is explored later.

Metagenomic approaches can also be applied to parasites. Cerebral toxoplasmosis has been traditionally challenging to diagnose because of the lack of biomarkers or nucleic acid in the CSF and relies on IgG, which has limited specificity and IgM which has limited sensitivity. Metagenomic sequencing

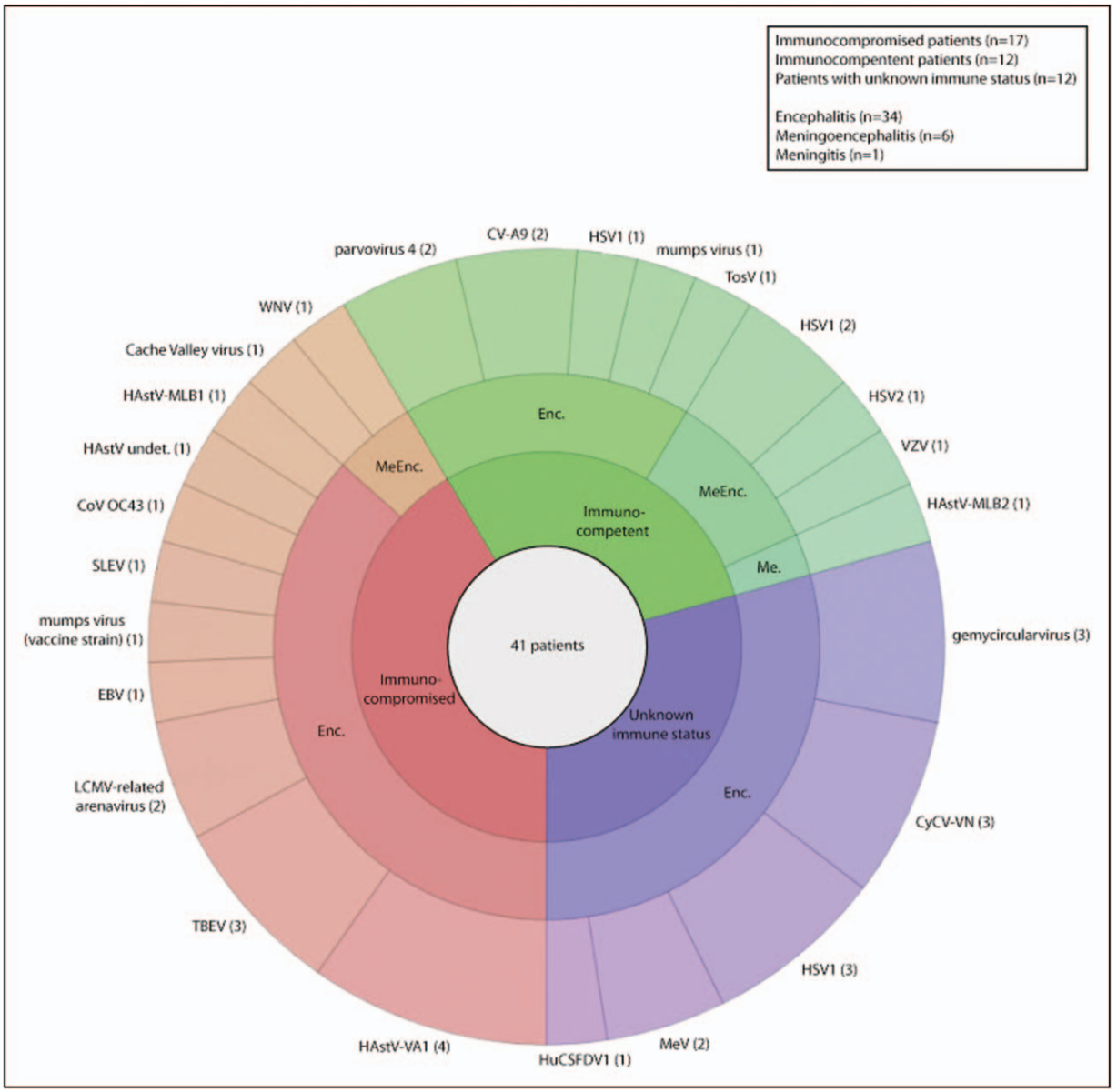

FIGURE 2. Summary of viruses identified using high-throughput sequencing (and / or subsequent confirmatory assays) in CSF and brain biopsy samples of 41 patients with suspected viral CNS infection, separated by immune status and clinical manifestations. Number of patients with suspected virus shown in brackets. CoV, coronavirus; CV-A9, coxsackie virus A9; CyCV-VN, cyclovirus Viet-Nam; EBV, Epstein–Barr virus; Enc., encephalitis; HAstV, human astrovirus; HSV, herpes simplex virus; HuCSFDV1, human CSF-associated densovirus 1; LCMV, lymphocytic choriomeningitis virus; Me., meningitis; MeEnc., meningoencephalitis; MeV, measles virus; SLEV, Saint Louis encephalitis virus; TBEV, tick-borne encephalitis virus; TosV, Toscana virus; undet., undetermined; VZV, varicella zoster virus; WNV, West Nile virus. Reproduced with permission [16▪▪].

identified toxoplasmosis DNA with 13% coverage of the genome in CSF from an HIV positive patient [19]. *Toxoplasma gondii*-specific PCR and Sanger sequencing were subsequently used to confirm the diagnosis. This recent report suggests an interesting new direction for toxoplasmosis diagnosis, but subsequent follow up should examine the sensitivity of the method and researchers should aim to use their results to identify targets for a more simple and specific diagnostic test.

The application of molecular diagnostics in the identification of viral CNS infections is often limited by the transient nature of viral RNA in the CSF, as in the case of Japanese encephalitis. The recent epidemic of the flavivirus Zika promoted the use of urine as a diagnostic specimen, particularly when detectable viraemia is absent [20]). Japanese encephalitis is similarly a flavivirus and the identification of Japanese encephalitis virus in urine in a patient with life-threatening encephalitis in Vietnam, in whom

multiple samples were tested and negative (CSF, stool and blood), illustrates the importance of expanding the range of specimens to which this technique can be applied [21].

Caution continues to be raised regarding the confirmation of pathogens identified using metagenomics which are suspected to be the cause of CNS syndromes, however [22]. In a recent study, 94 patients with chronic neuroinflammatory disorders thought to be noninfectious provided CSF samples for metagenomic analysis. Importantly, results were analysed using a weighted algorithm for the removal of sequences identified as environmental contaminants [23▪▪]. After the removal of these sequences, a causative organism was identified in seven patients; Taenia solium in two participants, HIV in one and fungi in four, findings highly likely to improve the clinical outcome for each patient. The careful consideration of environmental contaminants in patients with chronic neuropathological conditions is paramount, because of the historical erroneous detection of the reovirus XMRV in patients with chronic fatigue syndrome [24].

Human pegivirus has been detected in 1–45% of healthy blood donors worldwide [25]. Detection of this virus in CSF samples from patients with encephalitis through metagenomics sequencing brings questions related to its apparent pathogenicity versus coincidental detection from a leaky blood–brain barrier (BBB) [26]. A recent study used whole genome sequencing of pegivirus virions in samples from CSF and serum in patients with undiagnosed encephalitis [27] and demonstrated divergent sequences suggesting compartmentalization and potential pathological replication in the CNS. One further study found that the detection of pegivirus in the CSF was highly correlated with the detection of viraemia in patients with HIV, inferring that CNS detection was because of transfer across the BBB rather than replication in the CNS [28]. Pathogenicity question remains, however, and can likely only be answered through histopathological, immunological or novel transcriptomic analysis.

In addition to contamination risks and the risk of detecting nonpathogenic viruses, challenges for routine inclusion of metagenomic analysis of CSF in clinical settings include the longer turnaround time (minimum 2–5 days, but often longer) compared to PCR, higher cost and specialist laboratory and bioinformatic requirements. Counter to that, routine bacterial and fungal cultures have similar or longer turn-around times (2–5 days), and each of the listed limitations for metagenomics has significantly reduced over the past 5 years.

TRANSCRIPTOMICS AND PROTEOMICS

Aside from metagenomics, advances in technology have demonstrated the potential application of other – omics disciplines based on functional expression of gene activity. These include transcriptomics, involving the study of mRNA, and proteomics, the study of proteins. Notably, diagnostic tools for detection of mRNA and protein may be pathogen specific or used to detect the host response to infection.

Tools for the detection of mRNA are the same as those for genome analysis, and as such, the technology is well in hand. Nonetheless, it is recognized that mRNA is highly dynamic, affected by external perturbations, and mRNA as a biomarker is relatively easily degraded in the extracellular environment [29,30]. Equally, there are concerns that the host response may not be sufficiently unique for different infections to enable differentiation based on transcriptomics. To this end, there has been minimal investigation of the potential of mRNA testing for the diagnosis of CNS infections.

In contrast to nucleic acid, the fundamental proteins of underlying cellular processes are considered more robust targets for detection. Proteomics investigation depends on enzyme-linked immunosorbent assays, microarrays and increasingly, mass-spectrometry. In recent years, the role of MALDI-TOF has had a considerable impact on microbiology laboratories worldwide [31]. For CNS infection, mass-spectrometry, even when combined with PCR, has limited sensitivity for common viral infections, such as enterovirus [32]. The technique is consequently mostly used in bacterial infections causing bacteraemia with associated CNS infections, with additional optimization for direct detection from CSF [33]. In contrast, host-specific proteomic biomarker discovery remains a minimally explored field [34,35].

RAPID TB TESTING

We felt that it is important to summarize developments in molecular testing for CNS tuberculosis separately. The Cepheid GeneXpert system was launched in 2004 and has been endorsed by WHO since 2013. The automated closed-cartridge system enables nucleic acid amplification testing to detect MTB and Rifampicin resistance within 2 h. Over 90% of Rifampicin-resistant isolates are also Isoniazid resistant, and the technology has had major implications for the rapid identification of multidrug-resistant tuberculosis. The accessibility and speed of processing are impressive; however, there have been ongoing issues with low sensitivity of the

test in CSF, and there has been limited evidence of impact on patient outcome [36,37].

In March 2017, the WHO changed their recommended diagnostic test for testing samples, including cerebrospinal fluid, for *Mycobacterium tuberculosis* (MTB) to the Xpert MTB/RIF Ultra assay. The next-generation Xpert MTB/RIF Ultra assay allows a larger sample volume, with two extra molecular targets for MTB, leading to improved analytical sensitivity from 131 bacilli per ml sputum to 16 bacilli per ml. In a prospective study of 129 HIV patients in Uganda, the ultra assay detected 21 (95%) of 22 microbiologically proven cases of tuberculous meningitis, which was higher than either Xpert (45% 10/22; $P = 0{\cdot}001$) or culture (45%, 10/22; $P = 0{\cdot}003$) [38▪▪]. However, results for Rifampicin resistance (in samples in which there were sufficient bacilli) was only determined for 13 (62%). Although the findings are exciting, further work is needed to confirm results in other settings, and evaluate impact on patient outcomes. Case definitions and reference standards will also need to be updated accordingly. The ongoing challenge of negative results in pauci-bacillary TB meningitis is anticipated.

CONCLUSION

Significant improvements have been seen in the sensitivity, breadth, cost and speed of molecular diagnostic tests for meningitis and encephalitis over the past 5–10 years. The more recent past has brought the development and testing of several sensitive and specific panel diagnostics and, in the example of the Xpert MTB/RIF Ultra TB assay, has been included in global health policy recommendations. Metagenomic unbiased sequencing is being utilized in research settings, and increasingly for clinical cases where no pathogen has been identified, with the identification of novel viruses, and unexpected bacterial, viral and fungal causative pathogens and potentially nonpathogenic viruses. The important issue of contamination will require ongoing vigilance and improvement in technique and bioinformatics analysis. Steady progress is being made in reducing the number of gaps, and it is recognized that this involves different diagnostic approaches, as well as earlier and improved sampling of cases.

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Conflicts of interest

There are no conflicts of interest.

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- ▪▪ of outstanding interest

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