

Advances in molecular diagnostic testing for CNS 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 summarises 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 have revolutionised TB meningitis diagnostics with newly released recommendations for their use from the World Health Organisation.

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

CNS infections, molecular diagnostics, sequencing, PCR

Introduction

Current data demonstrate that even in the best-resourced centres, up to two thirds of cases of central nervous system 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 central nervous system 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 aetiologies.

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

PCR and nucleic acid detection methods

Due to 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 hours 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 revolutionised diagnostic facilities. Multiple commercial assays are now available (e.g. SeeGene, Fast Track Diagnostics). One of these, the Biofire FilmArray panel, requires minimal hands-on time and incorporates automated nucleic acid extraction, reverse transcription, amplification and results in a single machine in under an hour. A recent multi-centre 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/C. gattii* [5]. The disappointing performance of the assay for fungal detection highlights the importance of utilising additional simple and low-cost non-molecular 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 utilising 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 select 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 hybridisation. Microarrays for the detection of multiple CNS pathogens have been developed including one which targets 7 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 7 in which the bacterial culture was negative, the microarray detected a pathogen consistent with the clinical picture [7]. This positive finding required further optimisation 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 hospitalisation and duration of antibiotic treatment [8], strengthening the argument for further development. The challenge however, remains the high cost of single test cardruges (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 are 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 (cDNA) strand from each fragment of genomic material in a sample followed with bioinformatic analysis to firstly remove human sequences and assembly remaining fragments with comparison to publically available sequence databases (Figure 1). 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 16s (bacteria) and 18s (fungal), amongst others, followed by sequencing of the product for species identifications [10**]. 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 [11**–13]. Sequencing, of course, does not identify phenotypical antibacterial sensitivity and therefore offers only supplementary or complementary analysis in bacterial CNS infection.

Long-read sequencing utilises 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} meters 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 (~4hours 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 [14**]. 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 [15**]. In this review, studies which used metagenomic approaches identified potential viruses causing disease in 41 patients, 10 of which were unexpected (Figure 2). A lack of consistency in laboratory (platform, protocols) and bioinformatics analysis (pipelines, databases), and the lack of standardised negative controls samples is 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 have since emerged [16], in keeping with an exponential trend for the identification of

novel pathogens using metagenomics [17*]. 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 due to 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 identified toxoplasmosis DNA with 13% coverage of the genome in CSF from an HIV positive patient [18]. *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 tests.

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 (JE). The recent epidemic of the flavivirus Zika promoted the use of urine as a diagnostic specimen, particularly when detectable viraemia is absent [19]). JE is similarly a flavivirus and the identification of JE virus in urine in a patient with life-threatening encephalitis in Vietnam, in whom multiple samples were tested and negative (CSF, stool, blood) illustrates the importance of expanding the range of specimens to which this technique can be applied [20].

Caution continues to be raised regarding the confirmation of pathogens identified using metagenomics which are suspected to be the cause of CNS syndromes, however [21]. In a recent study, 94 patients with chronic neuroinflammatory disorders thought to be non-infectious provided CSF samples for metagenomic analysis. Importantly, results were analysed using a weighted algorithm for the removal of sequences identified as environmental contaminants [22**]. After removal of these sequences, a causative organism was identified in 7 patients; *Taenia solium* in 2 participants, HIV in one and fungi in 4, 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, due to the historical erroneous detection of the reovirus XMRV in patients with chronic fatigue syndrome [23].

Human pegivirus has been detected in 1-45% of healthy blood donors worldwide [24]. 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) [25]. A recent study used whole genome sequencing of pegivirus virions in samples from CSF and serum in patients with undiagnosed encephalitis [26] and demonstrated divergent sequences suggesting compartmentalisation and potential pathological replication in the CNS. One further study found that the detection of pegivirus in the CSF was highly correlated with detection of viraemia in patients with HIV, inferring that CNS detection was due to transfer across the BBB rather than replication in the CNS [27]. 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 non-pathogenic 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 have significantly reduced over the past 5 years.

Transcriptomics and proteomics

Aside from metagenomics, advances in technology have demonstrated 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 detection of mRNA are the same as those for genome analysis, and as such, the technology is well in hand. Nonetheless, it is recognised that mRNA is highly dynamic, affected by external perturbations, and mRNA as a biomarker is relatively easily degraded in the extracellular environment [28,29]. 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 [30]. For CNS infection, mass-spectrometry, even when combined with PCR has limited sensitivity for common viral infections such as enterovirus [31]. The technique is consequently mostly used in bacterial infections causing bacteraemia with associated CNS infections, with additional optimisation for direct detection from CSF [32]. In contrast, host-specific proteomic biomarker discovery remains a minimally explored field [33,34].

Rapid TB testing

We felt it important to summarise 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 hours. Over 90% of Rifampicin resistant isolates are also Isoniazid resistant, and the technology has had major implications for the rapid identification of multi-drug 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 [35,36].

In March 2017, the World Health Organisation (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; $p=0.001$) or culture (45%, 10/22; $p=0.003$) [37**]. 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, have been included in global health policy recommendations. Metagenomic unbiased sequencing is being utilised 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 non-pathogenic 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 recognised that this involves different diagnostic approaches, as well as earlier and improved sampling of cases.

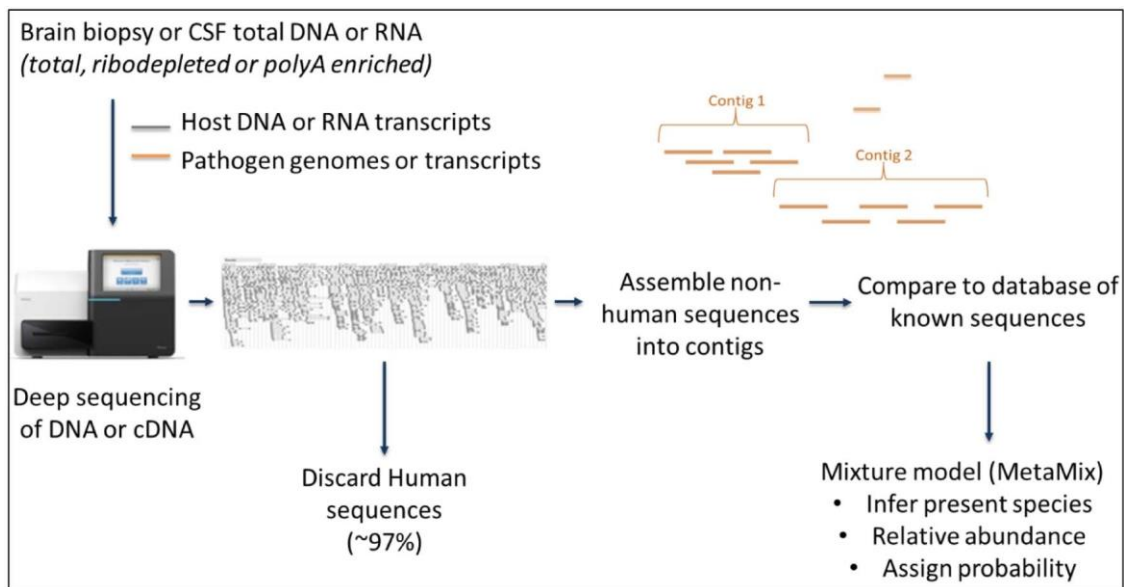


Figure 1. Illustration of typical metagenomics workflow (MetaMix is an example of an analysis tool (38))

Immunocompromised patients (n=17)
 Immunocompetent patients (n=12)
 Patients with unknown immune status (n=12)
 Encephalitis (n=34)
 Meningoencephalitis (n=6)
 Meningitis (n=1)

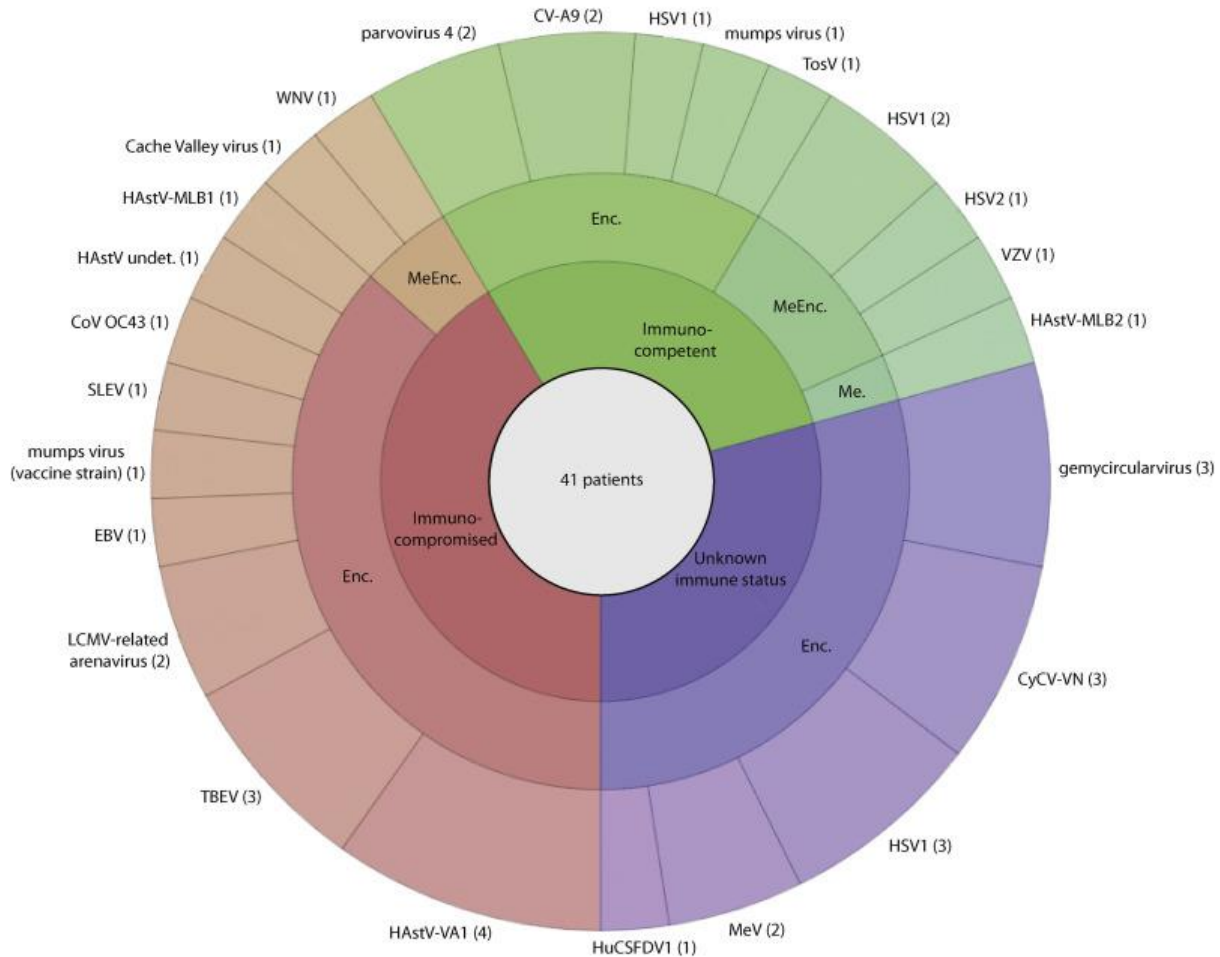


Figure 2. Reproduced with permission(15). Summary of viruses identified using high-throughput sequencing (and / or subsequent confirmatory assays) in CSF and brain biopsy samples of 41 patients with suspected viral CNS infection, separated by immune status and clinical manifestations. Number of patients with suspected virus shown in brackets. Enc., encephalitis; MeEnc., meningoencephalitis; Me., meningitis; CyCV-VN, cyclovirus Viet-Nam; HSV, herpes simplex virus; MeV, measles virus; HuCSFDV1, human CSF-associated densovirus 1; CV-A9, coxsackie virus A9; TosV, Toscana virus; VZV, varicella zoster virus; HAstV, human astrovirus; TBEV, tick-borne encephalitis virus; LCMV, lymphocytic choriomeningitis virus; EBV, Epstein Barr virus; SLEV, Saint Louis encephalitis virus; CoV, coronavirus; WNV, West Nile virus; undet., undetermined.

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Conflicts of interest

None

Key Bullet 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 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 non-pathogenic 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.

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*of special interest

**of outstanding interest

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