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ORIGINAL RESEARCH

High-throughput and Efficient Assay for Central Nervous System Infection with Targeted Nanopore Sequencing Technology

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Introduction: Central nervous system (CNS) infections represent a significant global public health concern and are characterized by high morbidity and mortality rates. In this study, we developed an integrated diagnostic approach for CNS infections by combining high-throughput nanopore sequencing with multiplex PCR amplification, designated targeted nanopore sequencing (tNPS).

Methods: The tNPS assay employed a dual detection strategy incorporating pathogen-specific primers targeting 17 prevalent CNS pathogens (seven bacteria, one fungus and nine DNA viruses), with universal primers for the comprehensive amplification of full-length 16S ribosomal RNA (16S rRNA) and internal transcribed spacer (ITS) regions.

Results: Analytical validation of tNPS was successfully carried out using the 12 positive reference strains (seven bacteria, one fungus, and four DNA viruses) individually, the ZymoBIOMICS microbial community (eight bacteria and two fungi), the laboratory synthetic community of bacteria and fungi (seven bacteria and one fungus), and the laboratory synthetic community of viruses (five DNA viruses). With accelerated turnaround time within 8 h, the tNPS also assayed 11 clinical cerebrospinal fluid (CSF) samples, which further confirmed the feasibility of precise identification of CNS pathogens compared to CSF culture and metagenomic next-generation sequencing.

Discussion: Our tNPS as a culture-independent diagnostic assay offered enhanced efficiency, high-throughput capability, and an expanded pathogen detection spectrum, facilitating potential implementation in molecular diagnosis of CNS infection.

Keywords: central nervous system infection, cerebrospinal fluid, assay development, nanopore sequencing, targeted sequencing, multiplex PCR

Introduction

Central nervous system (CNS) infections encompass neuroinflammatory conditions triggered by pathogenic invasion of bacteria, viruses, fungi, or parasites into the brain, spinal cord, or meninges, with meningitis, encephalitis, myelitis, and cerebral abscesses representing the predominant clinical manifestations.[1–3] As a critical global health priority, these infections are associated with substantial mortality and long-term neurological disability, compounded by diagnostic challenges in rapid pathogen identification.[3–6] Early implementation of targeted antimicrobial therapy within the first 6–8 h of symptom onset has been demonstrated to significantly improve clinical outcomes, underscoring the importance of precise diagnostic modalities.[7,8]

According to existing guidelines and systematic review,[1–3,6,9,10] the epidemiologically prevalent CNS pathogens addressed include bacterial agents, such as *Streptococcus pneumoniae, Neisseria meningitidis*, *Streptococcus agalactiae*, *Listeria monocytogenes*, *Haemophilus influenzae*, *Mycobacterium tuberculosis*, *Escherichia coli*; fungal pathogens, such

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as *Cryptococcus neoformans*; and neurotropic viruses, such as herpes simplex virus type 1 (HSV-1), herpes simplex virus type 2 (HSV-2), varicella-zoster virus (VZV), Epstein–Barr virus (EBV), cytomegalovirus (CMV), human herpesvirus 6 (HHV-6), human herpesvirus 7 (HHV-7), adenovirus (AdV), and JC polyomavirus (JCV).

Conventional diagnostic workflows in clinical microbiology laboratories predominantly rely on culture-based isolation (several days turnaround time), monoplex/multiplex qPCR assays (limited pre-defined targets), and serological profiling (IgM/IgG detection).[4,11,12] While these constitute current diagnostic standards, critical limitations persist: culture methods exhibit sensitivity reduction in antibiotic-pretreated patients, qPCR is restricted by low-plex capacity due to spectral overlap, serological tests suffer from high false-negative rates during early infection windows, and these approaches frequently fail to detect polymicrobial infections. To overcome these challenges, pathogen detection based on high-throughput next-generation sequencing (NGS), including metagenomic next-generation sequencing (mNGS) and targeted next-generation sequencing (tNGS), has been increasingly adopted in clinical practice.[5,6,13,14] And nanopore sequencing technology offers furthermore advantages, including real-time sequencing capability, ultralong read lengths, and rapid data analysis, and our previous studies, along with emerging evidence from other research groups, have demonstrated the feasibility and efficiency of this technology in detecting pathogens and their drug resistance.[8,15–22]

This study aimed to develop an improved targeted nanopore sequencing (tNPS) method for the specific detection of CNS pathogens. A dual-amplicon multiplex PCR strategy was introduced using specific primers targeting prevalent pathogens, along with universal primers for full-length 16S ribosomal RNA (16S rRNA) and internal transcribed spacer (ITS) regions, followed by nanopore sequencing and bioinformatic analysis. By enabling culture-independent diagnosis and expanded pathogen coverage as well as subsequent RNA workflow, our tNPS could potentially be used to assay CNS infections.

Materials and Methods

Microbial and Clinical Samples

Reference strains were procured from certified biological resource centers, including *E. coli, S. agalactiae, S. pneumoniae*, and *C. neoformans* from ATCC (Manassas, VA, USA); *M. tuberculosis*, N. *meningitidis, H. influenzae*, and *L. monocytogenes* from Xinyang Laiyao Biotechnology (Henan, China); HSV-1, HSV-2, and EBV from Bangdesheng Biotechnology (Guangdong, China); and CMV and VZV from the Key Laboratory of Laboratory Medicine at Wenzhou Medical University (Zhejiang, China).

With informed consent obtained from the study participants prior to study commencement, 11 cerebrospinal fluid (CSF) samples were retrospectively collected from patients with suspected CNS infections, which were approved by the Research Ethics Committee of the Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University in compliance with the Declaration of Helsinki (Ethics Approval No. 2024-K-108-01).

Construction of Plasmids

Three phylogenetically distinct organisms were selected, spanning bacteria, fungi, and DNA viruses: the extremely halophilic bacterium *Salinibacter ruber*, the soil-derived ascomycete *Trichoderma koningii*, and the plant-infecting *Cauliflower mosaic virus* (CaMV), which acted as internal controls because these are absent from the CSF microbiota under physiological conditions.[23,24] Target genes of these three internal controls were strategically chosen based on functional conservation and diagnostic utility: the DNA processing *dprA* gene from *S. ruber*, the cellobiohydrolase *cbh1* gene from *T. koningii*, and the viral coat protein *CaMVgp5* gene from CaMV. These sequences were codon-optimized, synthesized, and cloned into separate pUC57 plasmid backbones (GenScript Biotechnology, Nanjing, China).

Four neurotropic viruses associated with CNS infections underwent the same construction pipeline: AdV, HHV-6, HHV-7, and JCV. The diagnostic targets of the four viruses were selected based on conserved genomic regions: the AdV *Hexon* structural protein gene, HHV-6 *U90* immediate-early gene, HHV-7 *U36* tegument protein gene, and JCV *VP1* capsid gene.

DNA Extraction

Genomic DNA from the reference microbial and clinical samples used for tNPS detection was extracted using the boiling method, as described in our previous studies.[19,20] Briefly, 1 mL of sample liquid was centrifuged at 12,000 rpm for 10 min to remove the supernatant and resuspended in 50 µL of PBS, followed by the addition of 50 µL of nucleic acid extraction reagent (10 mM Tris-HCl, 1 mM EDTA, 1% TritonX-100, 1% NP-40, and 50% Chelex-100). The mixture was boiled at 100°C for 10 min and centrifuged at 12,000 rpm for 5 min, after which 10 µL of the supernatant was used as the template for PCR. Without RNA virus assayed in the current study, procedures for RNA extraction and reverse transcription were not included.

Mock Communities

The ZymoBIOMICS microbial community DNA standard D6306 (Zymo) was purchased (Zymo Research Corporation Irvine, CA) and contained genomic DNA of eight bacterial species (*L. monocytogenes, E. coli, Pseudomonas aeruginosa*, *Bacillus subtilis*, *Salmonella enterica*, *Lactobacillus fermentum*, *Enterococcus faecalis*, *Staphylococcus aureus*) and two fungal species (*Saccharomyces cerevisiae* and *C. neoformans*). Laboratory synthetic communities were established, consisting of a synthetic community of bacteria and fungi (SC-BF) containing genomic DNA from seven bacterial species (*E. coli, L. monocytogenes, S. pneumoniae, S. agalactiae, M. tuberculosis, H. influenzae*, and *N. meningitidis*), one fungal species (*C. neoformans*), and another synthetic community of viruses (SC-V), including genomic DNA from five DNA viruses (HSV-1, HSV-2, VZV, EBV, and CMV). These three communities were used as the mock samples (Supplementary Table S1).

Monoplex PCR and Sanger Sequencing

Pathogen-specific primers targeting the conserved genomic regions of 17 clinically relevant pathogens and three internal control plasmids were designed using NCBI Primer-BLAST with the following parameters: Tm, 60±3°C; GC content 40–60%, and amplicon size 200–1500 bp. Primer candidates underwent rigorous in silico validation through MFEprimer v. 4.0 (iGeneTech Bioscience, Beijing, China) with simulated reaction conditions (1.5 mM Mg^{2+}, 0.2 mM dNTPs). Pan-domain primers for the full-length regions of 16S rRNA (V1–V9, 1.5 kb) and ITS were also incorporated.

Following synthesis by GenScript Biotechnology, primer pairs were experimentally optimized using touchdown PCR (65–55°C gradient) to determine optimal concentrations (100–300 nM) and annealing temperatures. The target genes of the different pathogens were subjected to monoplex PCR and verified using Sanger sequencing. Sanger sequencing was performed using an ABI PRISM 3730 DNA Sequencer (Applied Biosystems, Foster, CA, USA), which was run by Shenggong Biotechnology (Shanghai, China).

Multiplex PCR of tNPS

The 25 µL multiplex PCR master mix contained 1× Q5 Hot-Start HiFi Buffer (New England Biolabs, Ipswich, MA, USA), 200 µM dNTPs, 0.2 µM each primer, 1× High GC enhancer, 0.5 U Q5 Hot-Start High-Fidelity DNA Polymerase (New England Biolabs), and 10 µL template DNA. Thermal cycling parameters were optimized as follows: initial denaturation at 98°C for 1 min; 35 cycles of denaturation at 95°C for 25 s; annealing at 57°C for 30 s; extension at 72°C for 3 min; and final extension at 72°C for 4 min.

To minimize primer interference, amplification was partitioned into two reaction panels: (1) bacterial-fungal panel: simultaneous detection of seven bacterial pathogens (*S. pneumoniae*, N. *meningitidis, E. coli, L. monocytogenes, M. tuberculosis, H. influenzae* and *S. agalactiae*) and one fungus (*C. neoformans*); and (2) viral panel, covering nine neurotropic DNA viruses (HSV-1, HSV-2, VZV, CMV, HHV-6, HHV-7, AdV, EBV, and JCV). After amplification, the products from both panels were pooled and assessed by 1% agarose gel electrophoresis (120 V, 120 min). Successful amplification was confirmed by visualization of the three internal control bands alongside pathogen-specific amplicons.

Library Preparation and Sequencing of tNPS

Amplicon quantification was performed using Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) with detection range 0.2–100 ng/μL on Qubit 4.0 Fluorometer, followed by magnetic bead purification with 1.2× AMPure XP Beads (Beckman Coulter, Pasadena, CA, USA) using 80% ethanol wash protocol. These amplicons were then mixed to create a library pool with equal amount of DNA, and library preparation strictly followed the manufacturer's specifications: end-repair/dA-tailing using NEBNext Ultra II Module (New England Biolabs) with 20-min incubation at 20°C, barcode ligation with Native Barcoding Kit 96 V14 (SQK-NBD114.96, Oxford Nanopore Technologies (ONT, Cambridge, UK) combined with Blunt/TA Ligase Master Mix (New England Biolabs) under 10-minute room temperature, and adapter ligation using Ligation Sequencing Kit SQK-LSK114 (ONT) with 15-min incubation at 25°C. All enzymatic reactions were terminated using 1.2× AMPure XP bead cleanups (Beckman Coulter) with dual ethanol washes (80%, v/v) and elution in 21 μL nuclease-free water.

Sequencing was performed on the PromethION P48 platform (ONT) equipped with R10.4.1 flow cells (FLO-PRO114M), with libraries loaded at 30–40 ng/library following ONT's voltage ramp protocol. Real-time sequencing was performed using the MinKNOW (v. 23.10.7) software for 1 h using Q20+ chemistry. Sequencing was performed by Benagen Technology (Hubei, China).

Bioinformatics and Statistical Analysis of tNPS

Real-time data acquisition was performed with MinKNOW and stored in the FAST5 file format, which was then converted into raw sequence data using Guppy (v. 6.5.7) base recognition analysis and stored in the FASTQ file format. There is a certain error rate during the sequencing process, and the Phred quality value can measure the probability of the incorrect detection of bases. Reads with a Phred quality value ≤9 (R10.4.1) were filtered out.

Following quality control procedures, Bowtie 2 (v. 2.5.4) was initially employed to filter out human-derived reads, thereby minimizing host background interference. Subsequently, the reads from each sample were aligned against the Kraken 2 prebuilt core_nt database (v. 12/28/2024) using Kraken 2 (v. 2.1.3) and the taxonomy of each read was assigned according to the taxonomic information of mapped subject sequence.[25–27] The core_nt database contained bacterial, archaeal, viral, and eukaryotic nucleotide sequences from GenBank and RefSeq, and were supplemented with annotated sequences from TPA and PDB (available at https://benlangmead.github.io/aws-indexes/k2). Bracken (v. 2.9) was then used to estimate species abundance and finally data visualization was performed using R (v. 4.3.2), Pavian (v. 1.2.1) and GraphPad (v. 9.5.1).[28–30]

The Student's *t*-test was used to calculate whether the samples were significantly different, with a *P*-value of <0.05.

CSF Culture for Clinical Samples

The CSF culture protocols included bacterial isolation on 5% sheep blood agar (BD Biosciences, San Jose, CA, USA) with 48-h incubation at 35°C and 5% CO_2, fungal screening via Sabouraud dextrose agar (Thermo Fisher Scientific) at 30°C for 72 h, and selective isolation of *H. influenzae* using chocolate *Haemophilus* agar (Thermo Fisher Scientific) under microaerophilic conditions. Pathogen identification in these CSF cultures was confirmed using MALDI-TOF mass spectrometry (Bruker BioSciences, Billerica, MA, USA).

mNGS for Clinical Samples

The mNGS testing protocols were as follows: nucleic acids extracted from CSF specimens were processed using the MGISP-100 automated library preparation system (BGI Genomics, Shenzhen, China) with dual-indexed adapters, sequencing was performed on the MGISEQ-2000 platform (BGI) generating 50 bp single-end reads, and raw data were automatically analyzed using the HALOS PMseq bioinformatics pipeline (BGI PMseq v. 3.0) incorporating human genome subtraction (GRCH38.p14), microbial database alignment (NCBI RefSeq v. 2023), and abundance reporting.

Results

Pipeline Establishment of tNPS

As shown in Figure 1A, the tNPS process for prevalent pathogens in CNS infections was established. Genomic DNA was extracted from the samples using the boiling method, and 17 pathogen target genes, 16S rRNA and ITS full-length gene

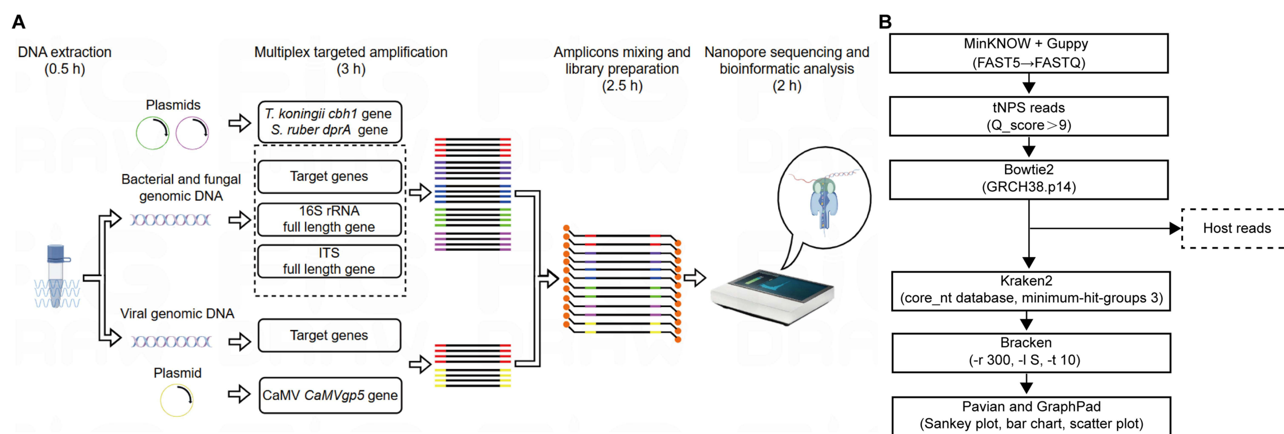

Figure 1 Workflow of tNPS-based experimental and bioinformatics analysis for detecting pathogens of CNS infection. (**A**) Genomic DNA was first extracted from samples using boiling method. Second, the target regions of genes related to 17 high-priority in CNS infections (seven bacteria, one fungus, and nine viruses), the full-length gene regions of 16S rRNA and ITS, and three internal control plasmids were then amplified by multiplex PCR in a bacterial-fungal tube and a viral tube. Third, the amplicons were mixed and pooled for library preparation. Fourth, the library was subjected to nanopore sequencing to generate long-read sequence data for bioinformatics analysis. The turnaround time was approximately 8 h. (**B**) MinKNOW first collected real-time data, which were converted to FASTQ format (raw reads) by Guppy. Second, Q_score filtered the raw reads into clean reads with base quality values >9. Third, using Bowtie 2, the human reads were removed by matching human genome reference sequence (GRCH38.p14). Kraken 2 was followed to map these reads to the core_nt database for species classification, with the parameter "–minimum-hit-groups" set to 3 to improve accuracy. Bracken also estimated the abundance of each taxon at the species level (with –l S), using a read length of 300 bp (the shortest read length in the dataset) and a threshold of 10 reads (with –t 10) to filter out low-abundance species and reduce noise. The results of bioinformatics analysis were finally visualized by Pavian and GraphPad.

regions, and three internal control plasmids were amplified by multiplex PCR in a bacterial-fungal tube and another virus tube. The amplicons from the two tubes were mixed for library preparation and nanopore sequencing to generate the long-read sequence data. Kraken 2 was used for the bioinformatics analysis (Figure 1B). The turnaround time of the tNPS was approximately 8 h.

Due to the wide variety of pathogens that cause CNS infections, 17 prevalent pathogens were selected as the targets of the assay, including seven bacteria (*S. pneumoniae*, N. *meningitidis, E. coli, L. monocytogenes, M. tuberculosis, H. influenzae* and *S. agalactiae*, with their corresponding *ply* pneumolysin gene, *ctrA* capsular transport protein A gene, *ppk* polyphosphate kinase gene, *hly* listeriolysin O gene, *rpoB* RNA polymerase β-subunit gene, *fucP* fucose permease gene and *pepF* oligopeptidase F gene), 1 fungus (*C. neoformans* with *FKS1* 1,3-β-D-glucan synthase gene), and nine DNA viruses (HSV-1, HSV-2, VZV, CMV, HHV-6, HHV-7, AdV, EBV, and JCV, with their corresponding *US3* serine/threonine protein kinase gene, *US39* tegument protein gene, *ORF38* tegument protein gene, *UL97* phosphotransferase gene, *U90* immediate-early gene, *U36* tegument protein gene, *Hexon* structural protein gene, *BFRF1* nuclear envelope protein gene and *VP1* capsid gene).

Sequencing Data Quality of tNPS

To evaluate the data quality of tNPS, 12 reference strains (*C. neoformans*, N. *meningitidis, L. monocytogenes, H. influenzae, S. agalactiae, E. coli, M. tuberculosis, S. pneumoniae*, HSV-1, HSV-2, VZV, and EBV) and mock communities (Zymo, SC-BF, and SC-V) samples were subjected to multiplex PCR using specific primers for target genes and universal primers for the full-length 16S and ITS genes. The amplicons were subjected to library preparation and nanopore sequencing to assess the quality of sequencing data.

After 1 h of sequencing of the reference strains, Zymo, SC-BF, and SC-V samples, the raw reads (65,565±9,132), clean reads (63,933±8,949), and non-human reads (63,871±9,222) were sufficient for subsequent microbial analysis, and the mean base quality of the clean reads was 17.20 (Figure 2, Table 1, and Supplementary Table S2). With an average classification rate of 95.05%, Kraken 2 accurately classified most of the microbial components into the correct species categories (Table 1).

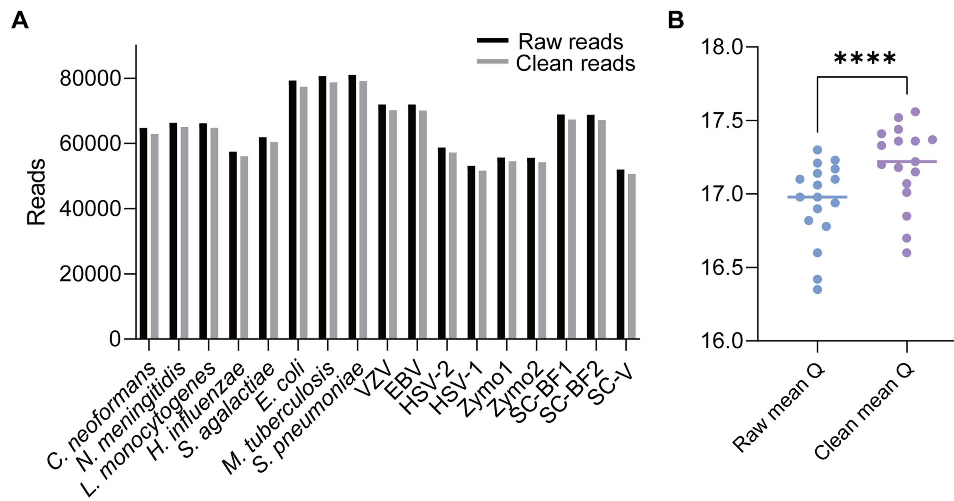

Figure 2 Data qualities of reference strains and mock communities using tNPS. (**A**) Raw and clean reads from 12 reference strains, Zymo (Zymo1 and Zymo2 were replicates), SC-BF (SC-BF1 and SC-BF2 were replicates), and SC-V samples. (**B**) Scatter plot comparing the mean base quality scores (Q) between raw and clean reads from ONT R10.4.1 sequencing platform. Mean (Q) raw reads = 16.95, clean reads = 17.20 (Student's *t*-test). ****P <0.0001.

Analytical Accuracy and Specificity of tNPS

As shown in Figure 3 and Supplementary Figure S1, the Sankey plots of Pavian illustrated that the 12 positive reference strains (seven bacteria, one fungus, and four DNA viruses) were accurately detected, with the distribution of species taxonomic abundance at the domain, phylum, genus, and species levels, which was also supported by electrophoretic patterns of multiplex PCR-based amplicons (Supplementary Figure S2A and B).

All 10 microorganisms (eight bacteria and two fungi) contained in Zymo, eight microorganisms (seven bacteria and one fungus) contained in SC-BF, and five microorganisms (five DNA viruses) contained in SC-V were further properly identified by tNPS (Figure 4), demonstrating that these tNPS assays had high accuracy and specificity in analytical

Table 1 Classification of Sequencing Data Using Kraken 2 and Core_nt Database

Type	Sample	Non-Human Reads	Classified Reads	Classified (%)	Unclassified Reads	Unclassified (%)
Fungus	*C. neoformans*	62,864	60,862	96.82	2,002	3.29
Bacteria	*N. meningitidis*	64,911	62,506	96.29	2,405	3.85
	L. monocytogenes	64,733	64,130	99.07	603	0.94
	H. influenzae	56,023	55,622	99.28	401	0.72
	S. agalactiae	60,390	58,532	96.92	1,858	3.17
	E. coli	77,344	74,678	96.55	2,666	3.57
	M. tuberculosis	78,678	77,901	99.01	777	1.00
	S. pneumoniae	79,056	78,182	98.89	874	1.12
Viruses	VZV	69,591	66,326	95.31	3,265	4.92
	EBV	70,060	61,046	87.13	9,014	14.77
	HSV-2	57,170	49,862	87.22	7,308	14.66
	HSV-1	51,587	42,039	81.49	9,548	22.71
Mock communities	Zymo1[a]	54,500	50,162	92.04	4,338	8.65
	Zymo2[a]	54,167	51,131	94.40	3,036	5.94
	SC-BF1[b]	67,293	66,916	99.44	377	0.56
	SC-BF2[b]	67,054	66,514	99.19	540	0.81
	SC-V	50,379	48,760	96.79	1,619	3.32

Notes: [a]Zymo1 and Zymo2 were replicates. [b]SC-BF1 and SC-BF2 were replicates.

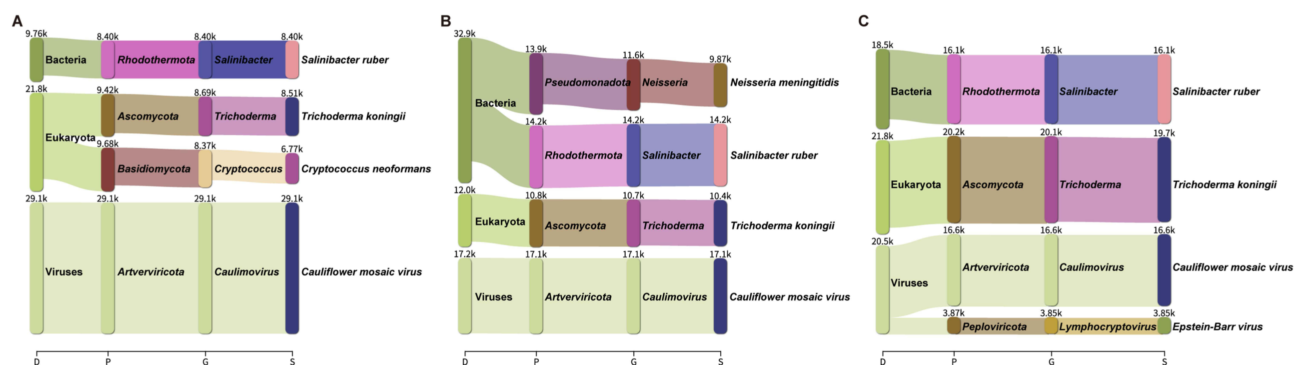

Figure 3 Sankey plots of tNPS results from reference strains. (**A**–**C**) *C. neoformans*, *N. meningitidis* and EBV, were identified along with the three internal controls (*S. ruber*, *T. koningii* and CaMV), respectively. Sankey plots of Pavian showed the distribution of species taxonomic abundance at the levels of D, domain; P, phylum; G, genus; and S, species. Different colors represented distinct classifications, and the width of the flow indicated their relative abundance.

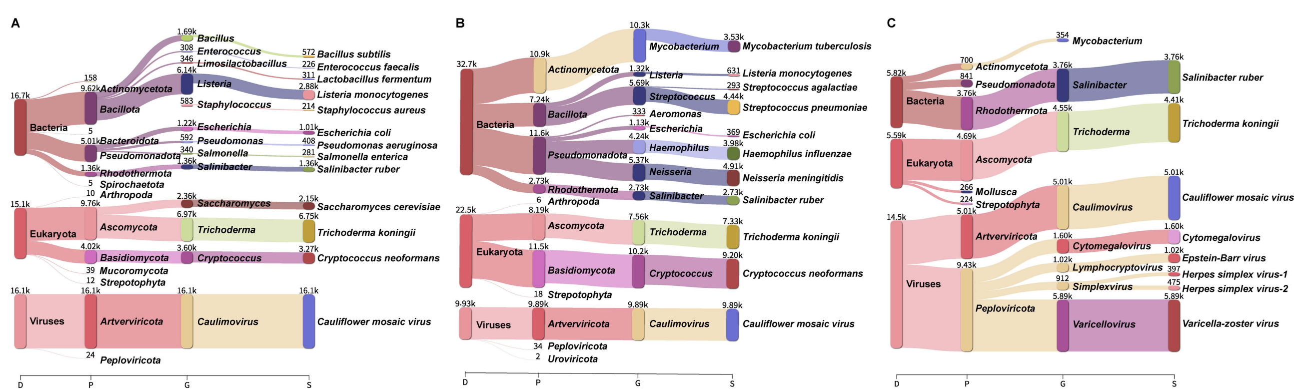

Figure 4 Sankey plots of tNPS results from mock communities. (**A**–**C**) With the respective data derived from assaying Zymo, SC-BF and SC-V, the three mock communities were identified along with the three internal controls (*S. ruber, T. koningii* and CaMV).
Abbreviations: D, domain; P, phylum; G, genus; S, species.

properties. Interestingly, the universal primer system (full-length 16S rRNA and ITS) provided an expanded pathogen detection spectrum at species-level resolution in Zymo (Figure 4A and Supplementary Table S1) and double-checked the identification process for bacteria and fungi (Supplementary Figure S2).

Analytical Detection Limit of tNPS

The limit of detection (LOD) was measured from eight representative pathogens (*S. pneumoniae*, N. *meningitidis, E. coli, L. monocytogenes, M. tuberculosis, H. influenzae, S. agalactiae*, and *C. neoformans*). Pathogens were diluted from 10^6 bacteria/mL in a series of 10-fold gradients to 10^2 bacteria/mL. The LOD was defined as the concentration at which the sequencing depth of each gene was greater than 100×. The results demonstrated that the LODs for *L. monocytogenes*, N. *meningitidis, M. tuberculosis* and *H. influenzae* were 10^2 bacteria/mL, while the others reached 10^3 bacteria/mL (Figure 3, Supplementary Figure S1, Figure S2C and D). Along with accuracy and specificity of tNPS, these analytical properties also confirmed that the optimal primer set were suitable for the dual-amplicon multiplex PCR strategy (Supplementary Table S3).

Microbial Classification and Reproducibility of tNPS

The Bray–Curtis dissimilarity analysis presented in Figure 5A showed distinct clustering patterns between Zymo replicates (Zymo1 and Zymo2) and synthetic community replicates (SC-BF1 and SC-BF2), validating robust within-group consistency and clear between-group differentiation.

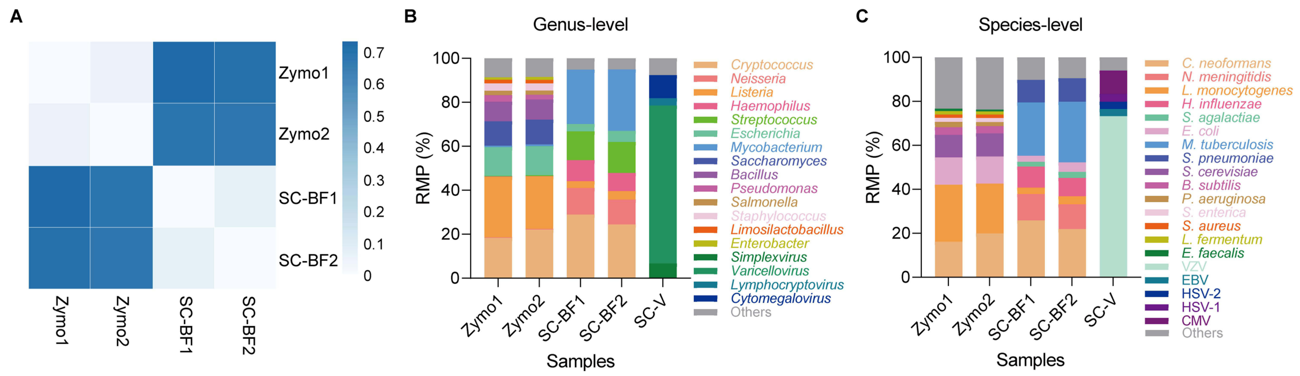

Figure 5 Microbial classification and reproducibility analysis of tNPS. (**A**) Heatmap of Bray–Curtis dissimilarities between Zymo replicates and SC-BF replicates. Color intensity represented relative compositional differences, with darker hues indicating higher divergence. (**B** and **C**) Microbial abundance ratios in Zymo (Zymo1 and Zymo2) and synthetic communities (SC-BF1, SC-BF2 and SC-V) at genus and species levels. Bar heights represented relative abundance ratios of microorganisms.

As shown in Figure 5B and C, the taxonomic classification of mock communities achieved high-resolution distinctions at both the genus and species levels, indicating that the sequencing depths and coverage of tNPS data were sufficiently resolved by bioinformatic analysis at the species level. In addition, these results confirmed that the reference core_nt database of the tNPS bioinformatics pipeline had sufficient integrity, and the accuracy of the alignment algorithm was reliable.

Clinical Application of tNPS

Eleven patients were enrolled in this retrospective study and their CSF clinical samples were subjected to CSF culture, mNGS and tNPS (Table 2). The multiplex PCR amplicons of these samples were visualized using agarose gel electrophoresis (Supplementary Figure S3). As CSF culture was only used for bacterial and fungal assays, their results showed that there was *S. pneumoniae* in one sample and no virus. As shown in Table 2 and Figure 6, tNPS identified the two positive samples as VZV and *S. pneumoniae*, and the others as negative, which was consistent with the results of mNGS. These patients with negative results were also found to be fever children with febrile seizures. VZV and *S. pneumoniae* were confirmed by monoplex PCR and Sanger sequencing (Table 2).

Table 2 Assay Results of Clinical Cerebrospinal Fluid (CSF) Samples

Sample	Gender	Age	CSF Culture	mNGS	tNPS	Sanger Sequencing
S01	Male	58	–	VZV	VZV	VZV
S02	Male	75	*S. pneumoniae*	*S. pneumoniae*	*S. pneumoniae*	*S. pneumoniae*
S03	Female	7	–	–	–	/
S04	Male	1	–	–	–	/
S05	Male	15	–	–	–	/
S06	Male	9	–	–	–	/
S07	Male	7	–	–	–	/
S08	Female	8	–	–	–	/
S09	Male	9	–	–	–	/
S10	Male	5	–	–	–	/
S11	Male	1	–	–	–	/

Notes: "–" indicates negative result. "/" indicates no test applied.

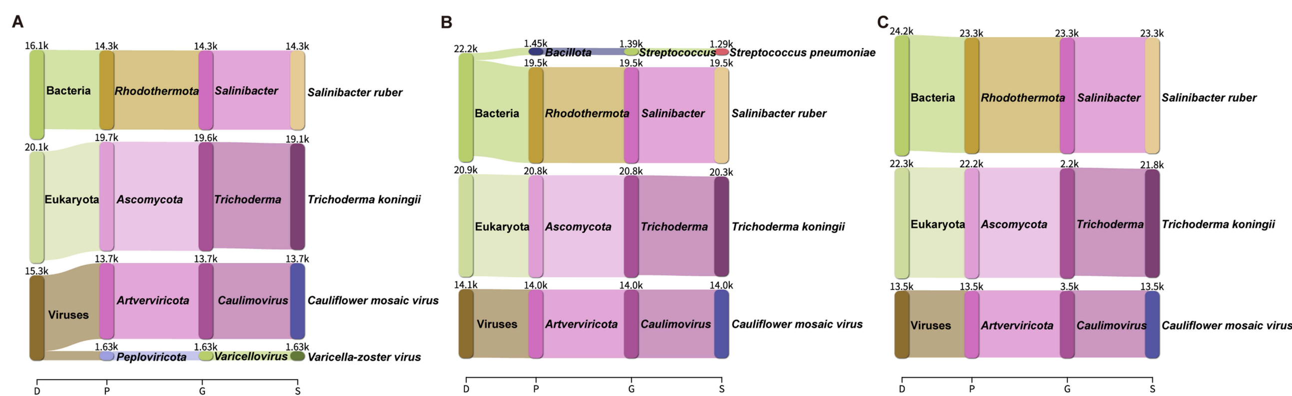

Figure 6 Sankey plots of tNPS results from clinical samples. (**A–C**) VZV, *S. pneumoniae* and negative result, were identified along with the three internal controls (*S. ruber, T. koningii* and CaMV), respectively.
Abbreviations: D, domain; P, phylum; G, genus; S, species.

Discussion

The tNPS assay in this study was successfully developed, which integrated a multitiered amplification strategy of pathogen-specific primers targeting 17 high-priority CNS pathogens joint pan-domain primers for full-length 16S rRNA and ITS, with real-time nanopore sequencing coupled with a Kraken2/Bracken bioinformatics pipeline. This method demonstrated superior diagnostic efficiency for CNS infections with a turnaround time of 8 h.

The selection of prevalent pathogens was based on multicenter epidemiological data from CNS infection cases, and 17 targeted pathogens (seven bacteria, one fungus, and nine DNA viruses) were included in this study, covering most of the clinically CNS-relevant pathogens.[1–3,6,9,10] Several sets of primers were initially screened and checked by the quality control tool MFEprimer, and the optimal primer set was experimentally validated as well as these primers' concentrations. The tNPS successfully assayed individual reference strains (seven bacteria, one fungus, and four DNA viruses) and microbial communities from Zymo (eight bacteria and two fungi), SC-BF (seven bacteria and one fungus) and SC-V (five DNA viruses), demonstrating its high specificity and accuracy. In addition, the LOD of tNPS was 10^3 bacteria/mL, with an optimal LOD of reaching10^2 bacteria/mL for *L. monocytogenes*, *N. meningitidis, M. tuberculosis* and *H. influenzae*. In addition, the assay results from Zymo confirmed the utility of 16S rRNA/ITS universal primers for the detection of other potential bacteria and fungi, as reported by the literature.[31–35] Therefore, the dual-amplicon multiplex PCR system of tNPS provides the opportunity for simultaneous detection of core pathogens and discovery of CNS co-infections.[1–3,6]

The clinical application of tNPS showed that, among 11 suspected patients, two were infected with VZV and *S. pneumoniae*, and the others were not infected by prevalent pathogens (febrile children with convulsions), which was consistent with the results of clinical mNGS.[5,6,14] Providing comprehensive pathogen identification within 8 h, tNPS could potentially enable timely and accurate diagnosis and medication of CNS infections.[3,4]

In recent decades, nanopore sequencing technology has demonstrated rapid advancements and remarkable advantages, with successful applications in pathogen diagnosis.[8,15–22] With ONT R10.4.1 flow cells dual-read-head architecture achieving single-molecule read accuracy, our previous studies effectively applied this technology to the analysis of drug-resistant genes in *M. tuberculosis*, validating its outstanding performance in microbial antimicrobial resistance testing.[19,20] This study extended the application of tNPS to pathogen detection in CNS infections, which further highlighted the key advantages of nanopore sequencing technology. The PromethION equipment could be flexibly replaced by portable MinION, Flongle, and other nanopore sequencing platforms from different suppliers.[17]

This study had some limitations. It had not yet included RNA viruses in the assay scope, which may affect the applicability for the identifying RNA pathogens. And the clinical sample size was small. For consolidation of tNPS performance, further investigation would be needed to optimize RNA workflow, expand pathogen type, increase clinical sample size, and especially conduct multicenter clinical trials.

Conclusion

As clinical evidence indicates that delayed or inappropriate treatment of CNS infections may result in severe complications and potentially life-threatening outcomes, our high-throughput and efficient tNPS approach had the potential to accelerate diagnosis, which would help clinicians achieve appropriate infection control and patient management.

Data Sharing Statement

The original contributions of this study are included in the article/supplementary material, and further inquiries can be directed to the corresponding authors.

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Disclosure

Prof. Dr Guangxin Xiang reports a patent “A kit for assaying central nervous system infection” pending to Wenzhou Medical University. The authors report no other conflicts of interest in this work.

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