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Clinical application of targeted nanopore sequencing in pathogen detection in patients with sepsis

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Abstract

Background Precision in detecting pathogens in sepsis patients is crucial for deploying targeted therapeutic strategies. The objective of the present investigation was to assess the efficacy of targeted nanopore sequencing (TNPseq) as a method for detecting sepsis pathogens.

Methods For this investigation, we stratified 90 patients with sepsis into 'improved' and 'unimproved' groups based on their clinical outcomes. Pathogenic microorganisms were detected in various sample types on the day of sepsis diagnosis, utilizing both conventional culture and TNPseq techniques. A comparative analysis of these methodologies was undertaken to assess their performance and efficacy in identifying pathogens in sepsis infections and discern pathogens with significant differences between the distinct patient groups.

Results The TNPseq analysis demonstrated superior performance in detecting pathogens in sepsis patients, achieving a significantly higher positivity rate (94.4%) compared to the culture method (30.0%, $p < 0.001$). TNPseq offered enhanced detection capabilities across a diverse array of pathogen types and sample types, outperforming culture particularly in the identification of fungi (35 vs. 8), viruses (46 vs. 1), and atypical pathogens. Both TNPseq and culture yielded concurrent positive results in 26 patient samples, yet TNPseq identified additional pathogens in 14 of these samples. A notable discrepancy in the types of infections was observed between the 'improved' and 'unimproved' groups, with a higher prevalence of single infections among the 'improving' cohort ($p < 0.05$). Significant differences in the incidence of *Acinetobacter baumannii* and *Clostridium striatum* were also evident between the two groups, underscoring the importance of focusing on these two pathogens in future clinical diagnostic efforts.

Conclusion This study underscores the versatility of TNPseq in identifying diverse pathogens in sepsis samples, ensuring its application as a diagnostic when pathogens elude conventional isolation or are difficult to culture. It particularly highlights the significant differences of *A. baumannii* and *C. striatum* in the two patient groups, pointing to potential research directions. TNPseq's potential in sepsis diagnosis is promising.

Keywords Sepsis, TNPseq, Culture, Diagnostic, Pathogenic microorganism

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Introduction

Sepsis, a severe condition marked by systemic inflammation from pathogenic microorganisms, can lead to septic shock and multiple organ failure and is a leading global cause of death [1]. Owing to the interaction of comorbidities, organ failure severity, pathogen variety, and the timing/effect of treatments, patients often face rare pathogens and intricate infections [2]. Thus, prompt sepsis diagnosis and rapid implementation of effective treatments are crucial for improving patient outcomes.

However, conventional culture for pathogen detection has substantial limitations, potentially undermining early sepsis diagnosis. For instance, conventional culture methods suffer from significant delays, often taking days to weeks for results, failing to meet the urgent need for rapid diagnosis and timely treatment [3]. Additionally, conventional culture methods are less sensitive and precise, and only detect known pathogens, missing unknown or hard-to-culture ones. This limitation can result in missed diagnoses and ineffective treatments for some sepsis cases [4].

Previous research has extensively employed metagenomic next-generation sequencing (mNGS) for the identification of pathogenic microorganisms, underscoring its clinical relevance [5, 6]. Nonetheless, the method has its challenges, including limited read length, high costs, long turnaround times, and difficulty in differentiating pathogens from commensal flora, which underscores the limitations of mNGS in clinical use [7]. These limitations highlight the necessity for progress in pathogen detection tech. Recently, targeted nanopore sequencing (TNPseq) has advanced, offering improved accuracy, longer read lengths, and better throughput for DNA/RNA sequencing. These breakthroughs have created new diagnostic pathways for sepsis, promising more precise and swift identification of infectious agents [8]. TNPseq for pathogens allows for quick detection, usually within hours, greatly decreasing turnaround time versus traditional culture methods. Furthermore, TNPseq is capable of identifying infectious agents that were previously undetected or challenging to culture [9]. For instance, Charalampous et al. have demonstrated that TNPseq is up to 96.6% more sensitive for pathogen detection compared to conventional culture methods [10]. The aforementioned advantages markedly boost sepsis detection sensitivity and accuracy, simplifying the diagnostic process. This innovation provides a groundbreaking method for early identification and treatment, advancing sepsis management.

In this study, we set out to evaluate the performance of conventional culture versus TNPseq in detecting pathogens in sepsis cases. We sampled two sepsis patient cohorts—those who improved and those who unimproved—and conducted both conventional cultures and TNPseq to identify pathogens. Furthermore, we analyzed pathogen discrepancies between the cohorts to pinpoint targets for clinical diagnosis and research emphasis. The outcomes will be correlated with TNPseq results to evaluate agreement and discrepancies between the methods. This comparative study aims to furnish empirical evidence and a conceptual basis for adopting TNPseq as a novel diagnostic and therapeutic tool in sepsis management.

Methods & materials

Study design

The study was conducted at Jingjiang People's Hospital. This study evaluated TNPseq's diagnostic capabilities, including sensitivity, specificity, and predictive values for detecting infectious agents. Furthermore, we compared it with standard culture methods, underlining its efficacy in promptly identifying pathogens and clinical diagnosis and medicine guidance for sepsis.

Inclusion criteria for the study required patients to have an illness onset within 24 h, ongoing or completed blood cultures within 12 h prior, a fever exceeding 38 °C, systemic signs of toxicity, and no clear focal point of infection; and patients were required to present with an infection that met sepsis diagnostic criteria, evidenced by a Sequential Organ Failure Assessment (SOFA) score of ≥ 2 [11]. Based on the laboratory test results of C-reactive protein (CRP) levels, procalcitonin (PCT) levels, serum creatinine (SCR), etc., and the judgment of the three experienced clinical experts, combined with their own experience, the patients with different final clinical outcomes were divided into two groups: "improved group" and "unimproved group". The "improved group" consisted of 64 individuals and the "unimproved group" consisted of 26 individuals.

Patients were ineligible for the study if they met any of the following exclusion criteria: inability to give informed consent; current renal replacement therapy for renal failure; extracorporeal membrane oxygenation for cardiac failure support.

It should be emphasized that the conduct of the study within a single hospital might impose constraints on the generalizability of the research outcomes [9].

Clinical data and sample collection

Clinical data, including demographics, medical history, lab results, treatments, and outcomes, were independently collected by experienced residents from medical records and meticulously reviewed. Trained professionals perform all sample collection and follows the following steps:

BALF: It is obtained utilizing a fiberbronchoscope at the level of the lung segment or sublung segment below the bronchus where the lesion is located, and is fully attracted and recovered after repeated irrigation with sterile normal saline. *Sputum:* Inhale sputum through the trachea or instruct the patient to perform deep sputum coughing with normal saline to complete the collection. *Cerebrospinal fluid:* Obtained by lumbar puncture, preferably after the second tube of cerebrospinal fluid, that is, the middle of the cerebrospinal fluid. *Tissue:* Collect solid tissue samples at the lesion, and collect at least 5 mm^3. *Peripheral blood:* cubital vein blood was obtained by intravenous puncture. *Pus:* Pus is drawn directly with a sterile syringe, and in cases requiring incision and drainage, pus is collected from the drainage material. *Pleural and abdominal fluid:* A professional aspirates fluid from the pleural and peritoneal cavity through a sterile puncture or surgical intervention.

Samples from infected sites were collected per protocol (≥ 3 mL; blood/BALF ≥ 5 mL), with three samples per patient: one for standard hospital culture and phenotypic susceptibility testing, one sent to Hangzhou Seq&Treat Biotechnology Co. Ltd for TNPseq, and one stored in the hospital's sample bank for potential future use by study reviewers, including pathogen isolation, Sanger sequencing, whole-genome sequencing, or PCR.

Conventional culture

The potential pathogens were routinely identified through standard culture methods [12]. Standard vitreous samples were cultured by inoculating them onto blood agar plates, which consist of tryptic soy agar supplemented with 5% sheep blood (Oxoid, Basingstoke, UK); chocolate agar enriched with 5% sheep blood, hemin, and vitamin K (Oxoid); Sabouraud agar (Becton Dickinson); and mercaptoacetic acid broth (Becton Dickinson). Each culture plate was sealed in an airtight bag or jar and subjected to anaerobic incubation at 35 °C for a period of 48 to 72 h, utilizing an anaerobic gas generating system. Thioglycylglycine broth was incubated at 35 °C for 24–48 h, and glucose agar plates at 25 °C for up to 30 days. Isolates were identified with biochemical assays.

DNA extraction

Clinical DNA extraction employed a magnetic bead protocol with mechanical lysis using 0.2-mm zirconia and 1-mm glass beads, homogenized at 55 Hz for 5 min using a Disruptor (Jingxin). After centrifugation, supernatant was treated with protease K, lysozyme, and GuSCN lysis buffer at 75 °C. Nucleic acids were bound to magnetic beads, washed with 80% ethanol, and eluted in TE buffer, quantified by Qubit 4.0 [13].

Library preparation and sequencing

Primers 27F/1492R and ITS1/4 were used to amplify 16S rRNA gene in bacteria and IST1/2 gene in fungi, respectively. Purified PCR amplicons underwent targeted nanopore barcode PCR via the PCR Barcoding Expansion Pack 1–96 protocol, involving a 95℃ denaturation for 3 min, 10 cycles of 95℃/15 s, 62℃/15 s, 65℃/1 min, and a final 65℃ extension for 1 min. Post-barcode PCR, products were purified with 0.6x AMPure beads, mixed in equal volumes, and the library was prepared with Ligation Sequencing Kit (SQK-LSK109), eluted in 15 µL TE buffer for quantification [14].

Purified nanopore libraries were sequenced on a MinION with R9.4.1 flow cell, loaded with 80 fmol following manufacturer's instructions. Real-time data were collected via ONT MinKNOW software, and the run was stopped after 30,000 reads/sample. Rejuvenated flow cells were prepared for reuse with the Flow Cell Wash Kit (EXP-WSH003), and to avoid carryover, barcode-labeled samples were not reused across sequencing batches.

Bioinformatics analysis

Since TNPseq is capable of detecting both live and dead microbial DNA, thereby complicating the differentiation among colonization, contamination, and active infections, a stringent filtration protocol for background microbes and contaminants was implemented by prior research [8]. Our bioinformatic analysis process was consistent with previous studies [15]. Briefly, the MinION data were transformed into FASTQ sequences with Guppy software. Subsequently, a comprehensive quality assessment and filtration process was carried out. Sequences that contained adapter and repetitive sequences, those with lengths either less than 200 bp or greater than 2000 bp, as well as sequences exhibiting a quality score Q lower than 7 were all removed. The remaining high-quality reads were then subjected to alignment using the Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information (NCBI). Microorganisms were classified as pathogens if they met the following criteria: having a relative abundance exceeding 0.5%, fulfilling a specific number of reads, and ranking within the Top 10. Specifically, the threshold for the number of specific reads was set at ≥ 3 reads for pathogenic bacteria and ≥ 1 read for fungi, viruses, and atypical pathogens such as *Mycobacterium* and *Nocardia* [9]. The sequencing outcomes will be further diagnostically evaluated by experienced clinical experts to finalize the filtering of background

microorganisms and contaminants. The details of the filtered microorganisms are presented in Table S1.

Statistical analysis

Statistical analyses were performed with SPSS v.24.0. Normally distributed data were presented as mean ± SD (X ± S), and non-normally distributed continuous data as median M (P25 ~ P75). Group comparisons used paired t-test, Mann–Whitney U test, or repeated measures ANOVA. Categorical data were described with frequency (n) or percentage (%) and compared using the chi-square test. A *p*-value of less than 0.05 was considered statistically significant.

Results

Characteristics of patients and samples

As indicated in Table 1, a total of 90 (63 men and 27 women; median age, 70 years) patients were ultimately included in this study, of whom 64 patients eventually improved and 26 did not. The underlying diseases prevalent among these patients included hypertension in 37 (43.5%) and diabetes mellitus in 20 (23.5%). Furthermore, as presented in Fig. 1, the majority of these patients presented with pulmonary infections ($n=30$, 33.3%), followed by pneumonia ($n=20$, 22.2%), bronchial infections ($n=10$, 11.1%), craniocerebral injuries ($n=5$, 5.6%), chronic obstructive pulmonary disease ($n=4$, 4.4%), pulmonary abscesses ($n=3$, 3.3%), pulmonary occupying lesions ($n=2$, 2.2%), central nervous system infections ($n=2$, 2.2%), and 12 others ($n=12$, 13.3%) including traumatic wet lung, acute gastroenteritis, and adenocarcinoma of the lung all in only 1 patient. Among the total samples, BALF predominated ($n=43$, 47.8%), followed by sputum ($n=19$, 21.1%), and cerebrospinal fluid ($n=7$, 7.8%). Other sample types included tissue ($n=8$, 8.9%), peripheral blood ($n=5$, 5.6%), pleural and abdominal effusion ($n=4$, 4.4%), pus ($n=3$, 3.3%), and puncture fluid ($n=1$, 1.2%).

Table 1 The basic clinical information of patients

	Improved group ($n=64$)	Unimproved group ($n=26$)
Men (%)	44 (68.8%)	19 (73.1%)
Women (%)	20 (34.4%)	7 (26.9%)
Median age (range)	67.5 (15–86)	72.5 (38–92)
Underlying condition (%)		
Hypertension	26 (40.6%)	11 (57.9%)
Diabetes mellitus	12 (18.8%)	8 (30.8%)
Neurologic disorder	6 (9.4%)	3 (11.5%)
Heart disease	5 (7.8%)	3 (11.5%)
Liver disease	7 (10.9%)	1 (3.8%)
Chronic kidney disease	1 (1.6%)	3 (11.5%)
Tuberculosis	3 (46.9%)	0 (0)
Stomach disease	0 (0)	2 (7.7%)
Others	6 (9.4%)	1 (3.8%)
Clinical indicators (range)		
SOFA score	3 (1–7)	3 (1–5)
APACHII score	19 (9–39)	17 (7–36)
Length of stay	19 (1–88)	22 (5–71)
Treatments (%)		
Mechanical ventilation	4 (6.3%)	17 (65.4%)
Vasoactive medications	2 (3.1%)	9 (34.6%)

Results for detecting pathogen

In this study, 90 patients with sepsis were analyzed using conventional culture methods and TNPseq to detect pathogens. Samples of each type were collected for culture before initiating antibiotic therapy. In all cases, the time interval between administering antibiotics and collecting TNPseq samples was within 24 h.

The results show that the sensitivity of microbial detection using TNPseq is improved by about 60%. Figure 2a illustrates that out of the 90 cases examined, 26 yielded positive results by both detection methods. A mere 2 cases exhibited complete concordance between the two techniques, accounting for 2.4% of the total. Additionally, the findings revealed a significantly higher positive detection rate for TNPseq compared to culture, with an increase of 64.4% (94.4% vs. 30.0%). As depicted in Fig. 2a, among the 90 samples, 26 were found to be positive by both TNPseq and culture. Only 2 of these cases showed complete agreement between the two methods, representing 2.2% of the total sample set. In 10 instances (11.1%), there was a complete discrepancy between the results of the two techniques. The results of 14 samples were "partially consistent" (15.6%), and in these samples, more pathogens were identified by TNPseq than by culture, which we also consider as "TNPseq + /culture-". Five samples yielded negative results across both methods. Notably, there was only 1 sample where a sample detection was positive by culture but negative with TNPseq (1.1%).

Figure 2b illustrates the pathogen types detected by TNPseq compared with the culture method. TNPseq successfully detected microorganisms in 85 out of 90 patients (94.4%), yielding a total of 58 unique microorganisms (comprising 31 bacterial, 9 fungal, 11 virals, and 8 atypical pathogens including *Nocardia*, *Chlamydia*, *Mycoplasma*, and *Tropheryma whippeli*), with 65 of these cases exhibiting polymicrobial infections. In contrast, conventional culture methods yielded positive results in only 27 out of 86 patients (30.2%). Compared with the culture method, TNPseq excels in the detection of a

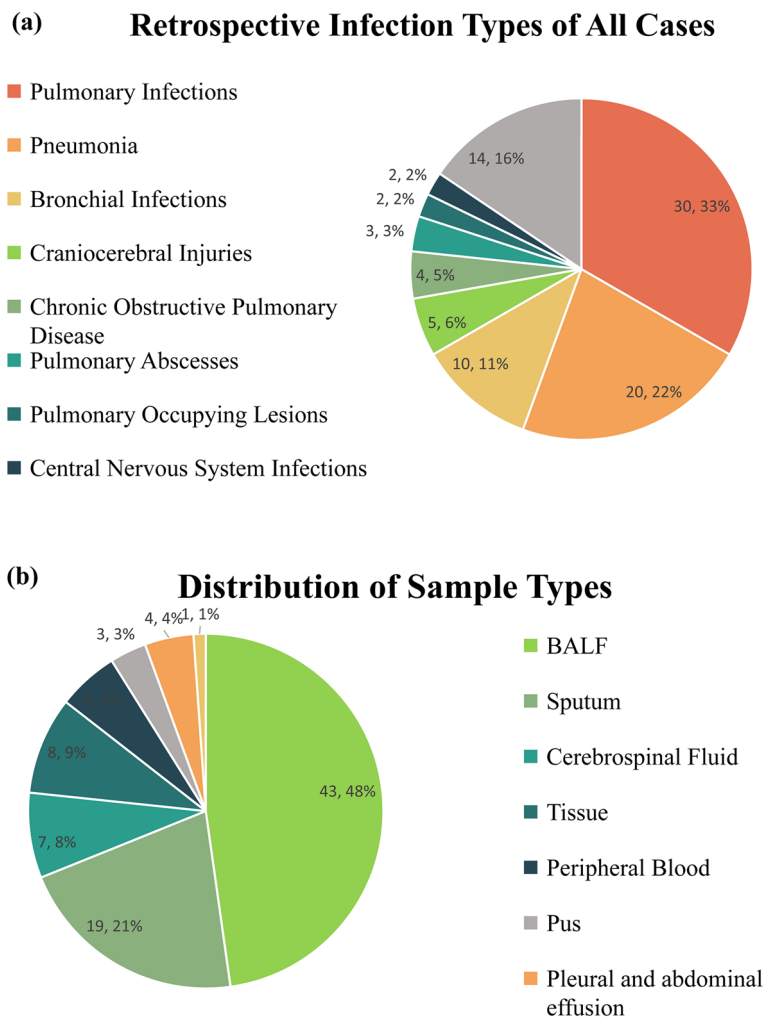

Fig. 1 Retrospective infections of patients and sample types

diverse array of microorganisms, including bacteria (58 vs. 19), fungi (32 vs. 7), and viruses (45 vs. 1). Furthermore, it is capable of detecting atypical pathogens, which are often missed by conventional culture methods.

Comparison of TNPseq and culture's detection performance

Figure 2c and Table 2 detail the pathogens identified outcomes by both TNPseq and conventional culture measurements. The most detected bacteria by both methods were *Acinetobacter baumannii* ($n=22$), followed by *Klebsiella pneumoniae* ($n=20$), *Pseudomonas aeruginosa* ($n=14$), and *Mycobacterium tuberculosis* ($n=12$), as well as *Stenotrophomonas maltophilia* ($n=11$) and *Corynebacterium striatum* ($n=9$). The most detected fungi were *Candida albicans* ($n=20$) and *Aspergillus fumigatus* ($n=17$). The conventional culture method barely detected any viral pathogens, with only a single sample positive for the *Human Herpesvirus 4* (*Epstein-Barr virus*, *EBV*). TNPseq, on the other hand, identified *EBV* as the most prevalent viral pathogen ($n=29$), followed by *Human Herpesvirus 1* (*HHV-1*) ($n=12$), *Human Herpesvirus 5* (*Cytomegalovirus*, *CMV*) ($n=6$), and *Human Herpesvirus 7* (*HHV-7*) ($n=6$) in the tested samples. Additionally, a sum of 46 microorganisms was detected by only TNPseq, while no pathogen was identified by only culture methods in isolation (Table S2). We conducted a comparative analysis of pathogen infections across patients exhibiting varying degrees of clinical improvement and uncovered significant differences in the prevalence of three key pathogens: *HHV-1* ($p=0.002$), *HHV-7* ($p=0.01$), *A. baumannii* ($p=0.001$), and *C. striatum* ($p=0.002$) between the distinct patient cohorts.

Figure 3 and Table S3 meticulously delineate the prevalence of pathogens identified through TNPseq and culture techniques within each sample and the types of

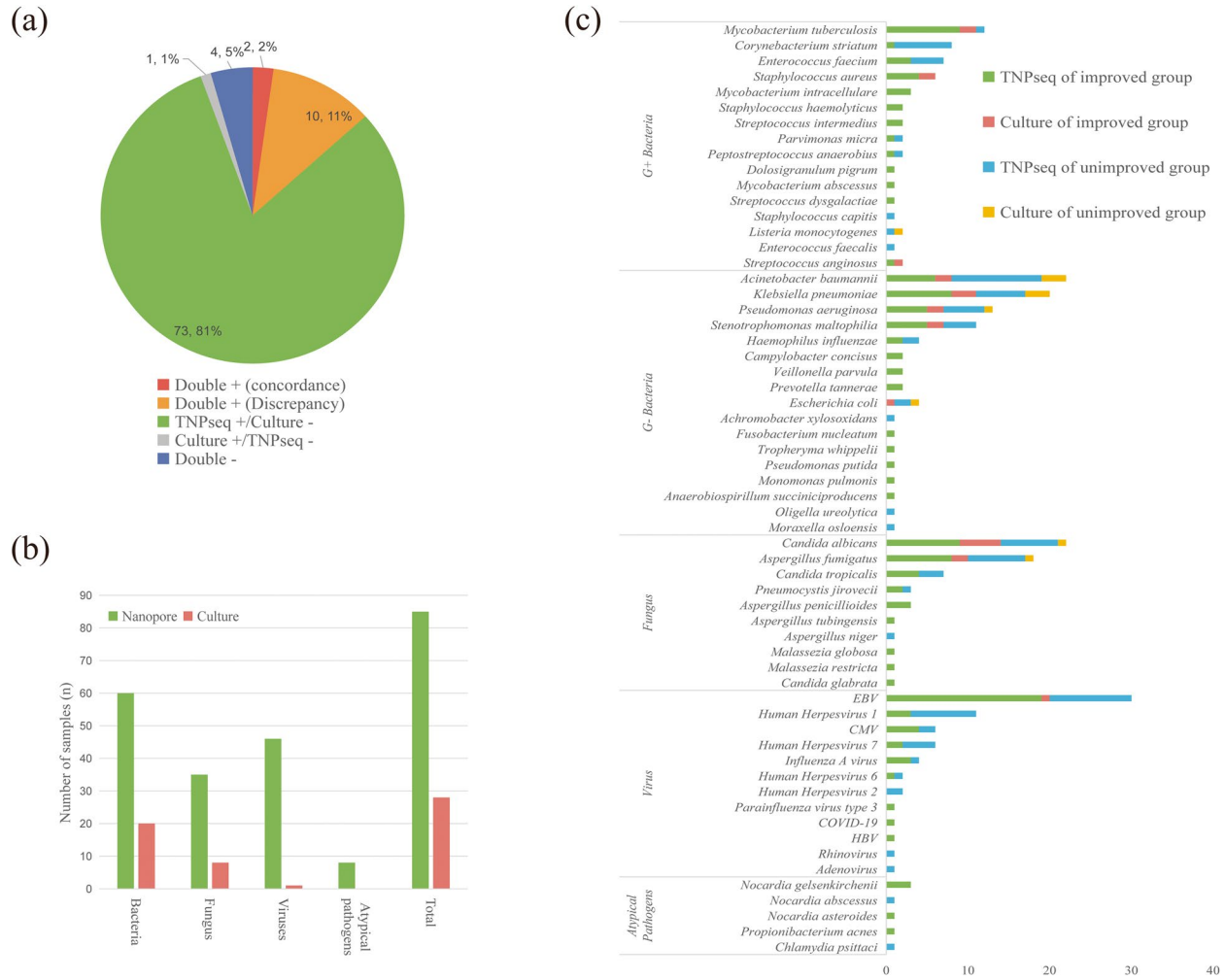

Fig. 2 TNPseq and culture detection results. **a** Positives and negatives detected by both methods. **b** The enumeration of pathogen types detected by both methods. **c** The detailed count of samples in which each pathogen was detected by the two methods

infection. The analysis of the dataset categorized by infection type revealed that a total of 67 patients were afflicted with mixed infections. Within this cohort, 44 patients (68.8%) belonged to the cohort that exhibited clinical improvement, contrasted with 24 patients (92.3%) in the cohort that did not show signs of improvement. This distinction between the two cohorts was statistically significant ($p=0.028$). Among the subset of 22 samples that presented with a single infection, the etiology was distributed as follows: 5 were infected by viral pathogens, 5 by fungal organisms, 11 by bacterial pathogens, and a single case by atypical pathogen.

Clinical effects on diagnosis and guidance of medicine

Based on the pathogen identification outcomes of TNPseq and their professional experience, clinical experts implemented targeted antibiotic therapies for all patients. For instance, in patient #5 where *A. baumannii* was detected, the experts chose Tigecycline and Biapenem for 14 and 21 days respectively, leading to a significant improvement in the patient's condition. In patient #6 with *Aspergillus penicillioides* infection, the antifungal drug voriconazole was administered, and the patient demonstrated clear recovery signs after 3 days. The majority of patients exhibited improvement with the targeted antibiotic treatments. The specific drug usage is detailed in Table S4. It is worth noting that due to restrictions in antiviral drug application, only one patient in this study took the antiviral drug Acyclovir and recovered after 19 days.

Laboratory tests of the two groups

In this investigation, we compared the laboratory findings between the final improved group and the unimproved group at the time of sepsis diagnosis. The analysis included a suite of biomarkers and tests: white blood cell

Table 2 Microorganisms identified by TNPseq and conventional culture

Pathogen Types	Pathogens	Number of TNPseq in improved group	Number of TNPseq in unimproved group	*P*-value	Number of culture in improved group	Number of culture in unimproved group
Virus	EBV	19	10	0.577	1	0
	Human Herpesvirus 1	3	8	**0.002**	0	0
	CMV	4	2	1	0	0
	Human Herpesvirus 7	2	4	**0.01**	0	0
	Influenza A virus	3	1	1	0	0
	Human Herpesvirus 6	1	1	1	0	0
	Human Herpesvirus 2	0	2	0.146	0	0
	Parainfluenza virus type 3	1	0	1	0	0
	COVID-19	1	0	1	0	0
	HBV	1	0	1	0	0
	Rhinovirus	0	2	0.146	0	0
	Adenovirus	0	1	0.64	0	0
Fungus	Candida albicans	9	7	0.253	3	1
	Aspergillus fumigatus	6	7	0.069	2	2
	Candida tropicalis	4	3	0.678	0	0
	Pneumocystis jirovecii	2	1	1	0	0
	Aspergillus penicillioides	3	0	0.635	0	0
	Aspergillus tubingensis	1	0	1	0	0
	Malassezia globosa	1	0	1	0	0
	Malassezia restricta	1	0	1	0	0
	Candida glabrata	1	0	1	0	0
	Aspergillus niger	0	1	0.64	0	0
G- Bacteria	Acinetobacter baumannii	6	11	**0.001**	2	3
	Klebsiella pneumoniae	8	6	0.35	3	3
	Pseudomonas aeruginosa	6	5	0.348	2	1
	Stenotrophomonas maltophilia	5	4	0.485	2	0
	Haemophilus influenzae	2	2	0.697	0	0
	Campylobacter concisus	2	0	0.902	0	0
	Veillonella parvula	2	0	0.902	0	0
	Prevotella tannerae	2	0	0.902	0	0
	Escherichia coli	0	2	0.146	1	1
	Fusobacterium nucleatum	1	0	1	0	0
	Pseudomonas putida	1	0	1	0	0
	Monomonas pulmonis	1	0	1	0	0
	Anaerobiospirillum succiniciproducens	1	0	1	0	0
	Moraxella osloensis	0	1	0.64	0	0
	Achromobacter xylosoxidans	0	1	0.64	0	0
	Oligella ureolytica	0	1	0.64	0	0

Table 2 (continued)

Pathogen Types	Pathogens	Number of TNPseq in improved group	Number of TNPseq in unimproved group	*P*-value	Number of culture in improved group	Number of culture in unimproved group
G + Bacteria	Mycobacterium tuberculosis	9	1	0.304	2	0
	Corynebacterium striatum	2	7	**0.002**	0	0
	Enterococcus faecium	3	4	0.199	0	0
	Staphylococcus aureus	4	0	0.459	2	0
	Mycobacterium intracellulare	3	0	0.365	0	0
	Staphylococcus haemolyticus	2	0	0.902	0	0
	Streptococcus intermedius	2	0	0.902	0	0
	Mycobacterium abscessus	2	0	0.902	0	0
	Parvimonas micra	1	1	1	0	0
	Peptostreptococcus anaerobius	1	1	1	0	0
	Streptococcus anginosus	1	0	1	1	0
	Dolosigranulum pigrum	1	0	1	0	0
	Streptococcus dysgalactiae	1	0	1	0	0
	Staphylococcus capitis	0	1	0.64	0	0
	Listeria monocytogenes	0	1	0.64	0	1
	Enterococcus faecalis	0	1	0.64	0	0
	Propionibacterium acnes	1	0	1	0	0
Atypical pathogens	Nocardia gelsenkirchenii	3	0	0.365	0	0
	Nocardia abscessus	0	1	0.64	0	0
	Nocardia asteroides	1	0	1	0	0
	Chlamydia psittaci	0	1	0.64	0	0
	Mycoplasma Pneumonia	2	0	0.902	0	0
	Tropheryma whippeli	1	0	1	0	0

(WBC) count, neutrophil count, lymphocyte count, CRP levels, PCT levels, SCR, total blood bilirubin (TBIL), and prothrombin time (PT). The findings revealed that the improved group exhibited significantly lower values across nearly all biomarker indicators on the day of sepsis diagnosis when compared to the unimproved group. The disparities in neutrophil count, lymphocyte count, CRP levels, PCT levels, SCR, and PT between the two groups were statistically significant (Table S5). All physiological parameters were assessed on the day of clinical diagnosis.

Discussion

In the present investigation, we evaluated the efficacy of TNPseq alongside conventional culture techniques for the identification of microbial agents in sepsis patients, especially for patients with different improvement profiles. Our dataset comprised samples from 90 sepsis patients, each subjected to detection using TNPseq and conventional culture methods. The sample types encompassed BALF, sputum, cerebrospinal fluid, tissue biopsies, peripheral blood, pleural and abdominal effusions, pus, and puncture fluids. The results showed that the sensitivity of TNPseq was higher than that of conventional culture methods.

Prior research shows TNPseq surpasses conventional pathology and culture for speed and sensitivity, especially in detecting *M. tuberculosis*, *Nocardia*, as well as viral and fungal pathogens [8, 9]. According to the findings of Luo et al., TNPseq enhances the detection of pathogens in lower respiratory tract infections,

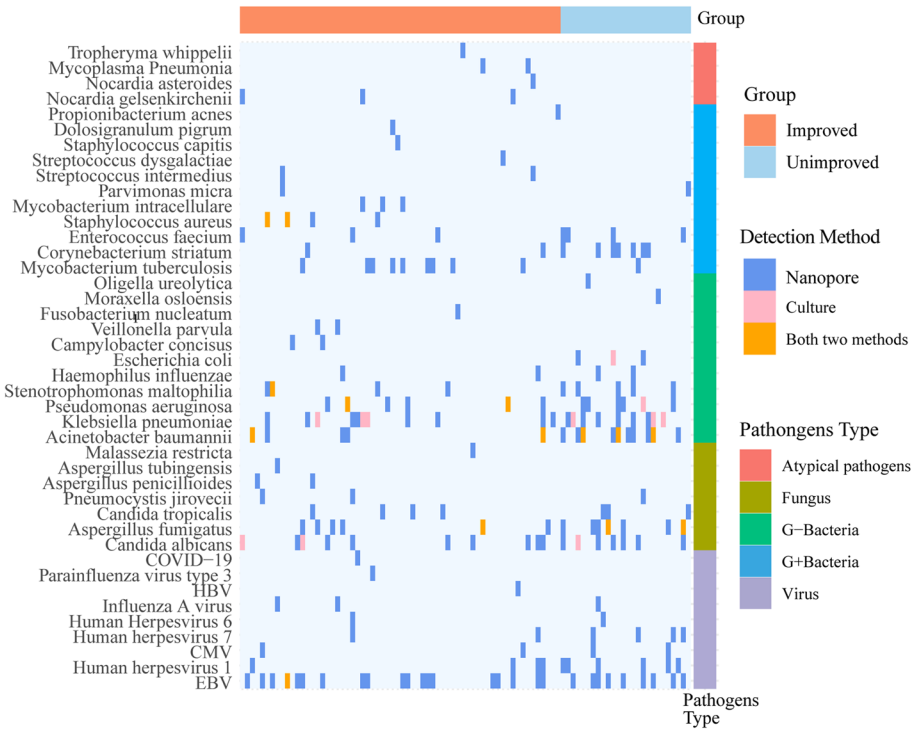

Fig. 3 Infection of each pathogen in each sample. The analysis includes only those pathogens detected in two or more samples, in addition to those that influence the statistics related to single and mixed infections. The horizontal axis represents individual patient samples, with the color bar at the top denoting the patient groups, orange represents the improved group and light blue represents the unimproved group. The vertical axis corresponds to each pathogen, and the color-coded legend on the right side indicates the distinct pathogen types to which it belongs, pink for atypical pathogens, grass green for fungi, turquoise for G- bacteria, aquamarine for G + bacteria, and lilac for viruses. Each cell within the heat map signifies the specific pathogen infection detected by diverse detection methods in a given patient sample, dark blue for Nanopore (TNPseq), light pink for culture, and bright orange for both two methods. For instance, the blue cell in the first row indicates that TNPseq detected the infection of the atypical pathogen Tropheryma whippelii in an improved patient sample

significantly abbreviating the reporting time from a conventional three days to a mere six hours [16]. The research conducted by Fu et al. has further shown that targeted TNPseq exhibits superior sensitivity to conventional culture-based methods for the diagnosis of infectious diseases, with a notable increase in detection rates (94.5% compared to 26.7%). This advancement is particularly pronounced in identifying pathogens in pleural fluid, ascites, BALF, urine, and wound secretions, reducing the detection time to a remarkably brief duration of 8 to 14 h [13]. Our results confirm prior evidence that TNPseq exhibits significantly higher sensitivity than culture (94.4% vs. 31.1%), across various sample types. There was suboptimal concordance between TNPseq and culture, with 10 of 12 samples positive by both methods showing pathogens undetected by TNPseq. However, this observation does not necessarily imply a high rate of false negatives with the TNPseq approach. Among these cultured pathogens, five were identified as *K. pneumoniae* and three as *C. albicans*. *K. pneumoniae* is a prevalent hospital-acquired bacterium that is susceptible to culture contamination, and *C. albicans* presents a challenge due to its robust cell wall, which can lead to insufficient read counts for detection as a pathogen [17, 18]. In the three samples containing *C. albicans*, the read counts were 1, 1, and 4, respectively.

Significant differences in the occurrence of *HHV-1*, *HHV-7*, *A. baumannii*, and *C. striatum* were noted among patients with diverse clinical recovery patterns. Despite not being primary sepsis agents, *HHV-1* and *HHV-7* can exacerbate illness in immunocompromised individuals, potentially increasing the risk of subsequent bacterial infections and indirect sepsis development [19]. *A. baumannii* stands as a pivotal pathogen in the etiology of sepsis, with its remarkable resilience to a spectrum of antibiotics conferring an ability to persist within healthcare settings [20]. The pathogen's ability to persist on hospital surfaces and equipment, along with its inter-patient transmission potential, poses a particular threat to immunocompromised patients, those with prolonged hospital stays, and users of invasive devices [21]. TNPseq detected *A. baumannii* in 17 samples, second only to

EBV. The improved patient group had 6 cases (35.3%) compared to 11 (64.7%) in the unimproved group, with 2 deaths accounting for half of all mortality. Similarly, *C. striatum* emerged as a key sepsis pathogen [22]. In our scope of this study, *C. striatum* was detected in 9 clinical samples, with 7 exhibiting no clinical improvement and one patient, co-infected with *A. baumannii*, succumbing to the infection. We suspect that these two pathogens are significant in causing sepsis, and their co-infection may exacerbate the disease. Nevertheless, our findings neither directly designate these pathogens as marker microbes nor perform mechanistic investigations on this hypothesis. The future diagnosis and treatment of sepsis might concentrate on these pathogens.

An additional benefit of TNPseq in sepsis pathogen detection is its ability to detect microorganisms typically missed by standard methods. For fungal infections, culturing is not only time-consuming and laborious but also requires pure isolates with spore production and identifiable characteristics for macroscopic and microscopic recognition [23]. Conversely, TNPseq exhibited improved specificity for detecting fungal pathogens in samples, allowing precise species-level identification [8, 9]. TNPseq identified fungal pathogens in 35 patients, surpassing culture-based detection in 8 patients, offering a valuable adjunct to the culture-based method. Furthermore, TNPseq detected fungi such as *Pneumocystis jirovecii* and various *Aspergillus* species, which are difficult to culture and often missed by conventional methods limited to common pathogens like *C. albicans* and *A. fumigatus*.

Conventional culture techniques for pathogens such as *Chlamydia*, *Nocardia*, *Mycoplasma*, and *Whipple*'s atypical bacteria are challenging, and routine molecular assays also have limitations that restrict their detection [24]. As a zoonotic pathogen transmitted from avian species to humans, *Chlamydia* psittaci is a significant cause of pneumonia. Wang et al. have harnessed the power of TNPseq to analyze bronchoalveolar lavage fluid samples, to facilitate the diagnosis of sepsis induced by *Chlamydia psittaci*, emphasizing the pivotal role of TNPseq in this diagnostic process [25]. *Nocardia* is also a key sepsis-inducing pathogen with a severe infection and high mortality rate. Its detection is challenging, as standard culture methods are inadequate for precise identification, thus requiring a rapid and reliable detection method [18, 26]. Our study found two cases of *Mycoplasma pneumoniae* and one of *T. whipplei*, which, though not typically sepsis initiators, may significantly contribute to sepsis in patients [19, 27]. Moreover, the detection of *T. whipplei* in a single infection underscores its potential as a direct sepsis trigger. TNPseq significantly outperforms standard diagnostics in detecting viruses often overlooked in Chinese sepsis protocols, identifying the virus in 46 patients compared to only one by culture.

Our study's results show that TNPseq produced five negative results, including one false negative for *K. pneumoniae*, which was culture-confirmed. Beyond the potential contamination aforementioned, this discrepancy could stem from insufficient bacterial material, technical errors during DNA extraction, or potential contamination [17]. For example, a possible failure to efficiently disrupt cell wall of *K. pneumonia* might hinder DNA release, thus affecting the sequencing process's electrical conductivity [28]. Technical glitches, like DNA degradation resulting from improper sample preservation, can likewise give rise to false negative outcomes. These limitations are controllable yet inevitable, and taking these elements into account will render TNPseq's test results more rational. Additionally, the absence of a standardized and universal method to distinguish between true pathogens, opportunistic colonizers, and contaminants is a persistent challenge [29]. To mitigate these issues, strict screening criteria are essential, especially for low-biomass samples, to avoid misidentifying lab contaminants and minimize cross-contamination from high-positive samples. The inclusion of negative controls is required at specimen preparation and sequencing stages to maintain result integrity [30]. It must be emphasized that although TNPseq can detect resistance genes and mutation sites in pathogens, comprehensive antimicrobial susceptibility profiling still requires extensive culture-based testing due to the diverse resistance mechanisms that pathogens can exhibit, with culture methods being vital for detailed drug sensitivity analysis [31].

Our study has several limitations. First, given that our sample grouping was based on patients' final disease outcomes, an imbalance in patient distribution between the two groups ensued. This imbalance restricts statistical power and precludes comprehensive subgroup analysis. Secondly, there is not always a match between TNPseq and culture samples among patients, which can give rise to substantial bias in the results. For example, certain patients used sputum samples for TNPseq while having blood cultures. This might have led to TNPseq detecting a large quantity of orally colonized bacteria, and only pathogens in the blood being cultured. This could potentially be one of the factors contributing to the lack of agreement between TNPseq and the outcomes of the culture method. Thirdly, our experimental design is based on previous findings to validate the rationality of this study. However, if it could be integrated with other advanced molecular techniques like mNGS as a reference standard, TNPseq would better position in the broader diagnostic domain [15, 17].

Conclusion

In conclusion, this study highlights the utility of TNPseq for detecting a range of pathogens in various sepsis samples, advocating its use as a diagnostic tool when culturing methods fall short or pathogens are not isolatable by conventional techniques. Notably, it reveals distinct differences between *A. baumannii* and *C. striatum* across patient outcomes, suggesting avenues for future inquiry. Despite requiring robust sample quality, TNPseq's clinical application and future role in sepsis diagnosis hold significant promise.

Abbreviations

TNPseq	Targeted nanopore sequencing
mNGS	Metagenomic next-generation sequencing
BALF	Bronchoalveolar lavage fluid
SOFA	Sequential Organ Failure Assessment
EBV	*Human Herpesvirus 4* (*Epstein-Barr virus*)
HHV-1	*Human Herpesvirus 1*
CMV	*Human Herpesvirus 5* (*Cytomegalovirus*)
HHV-7	*Human Herpesvirus 7*
WBC	White blood cell
CRP	C-reactive protein
PCT	Procalcitonin
SCR	Serum creatinine
TBIL	Total blood bilirubin
PT	Prothrombin time

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

Supplementary Material 4.

Supplementary Material 5.

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Authors' contributions

M. C. contributed to the study design. L. B. and Y. Q. collected the data and conducted laboratory testing. Y. C., J. Z., and Y. L. helped with the bioinformatics analysis. M. C. occupied the initial manuscript drafting and revisions, L. B. and X. L. calibrated the data analysis, and finally prepared the manuscript. Funding for this study was provided by Y. L. All the authors read and approved the final manuscript.

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Data availability

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2021) in National Genomics Data Center (Nucleic Acids Res 2024), China National Center for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: CRA019860) that are publicly accessible at https://ngdc.cncb.ac.cn/gsa.

Declarations

Ethics approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki. The research protocol was scrutinized and greenlit by the Ethics Committee of Jingjiang People's Hospital (reference number 24-KY-038–01). Written informed consent was secured from all participants or their legally appointed guardians before their inclusion in the study.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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