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The value of metagenomic next-generation sequencing in the diagnosis of fever of unknown origin

Lan Min Lai[1,2], Qing-gen Chen[1,2], Yang Liu[1,2], Rui Zhao[1], Mei Ling Cao[1] & Lei Yuan[1]✉

Fever of unknown origin (FUO) caused by infection is a disease state characterized by complex pathogens and remains a diagnostic dilemma. Metagenomic next-generation sequencing (mNGS) technology is a promising diagnostic tool for identifying pathogenic microbes of FUO caused by infection. Little is known about the clinical impact of mNGS in the etiological diagnosis of FUO. This study focuses on the value of mNGS in the etiologic diagnosis of FUO by diagnostic performance, further clarifying the value of mNGS in clinical management. In a single-centre retrospective cohort study, 263 FUO patients who underwent both mNGS and culture at the First Affiliated Hospital of Nanchang University were enrolled from December 2020 to February 2023. The sensitivity and specificity of culture and mNGS were analyzed based on the final clinical diagnosis as the gold standard to assess the diagnostic value of mNGS in FUO cases. Among the 263 patients, 69.96%(184/263) cases were diagnosed as infectious diseases, of which lower respiratory tract infections were the most common, accounting for 53.26%(98/184). 30.04%(79/263) cases had a diagnosis of non-infectious disease. From these cases, mNGS identified 150 true-positive cases, 21 false-positive cases, 58 true-negative cases, and 34 false-negative cases. The sensitivity of mNGS in infection diagnosis was much higher than that of culture [81.52%(150/184) vs. 47.28%(87/184)], but the specificity was the opposite[73.42%(58/79) vs. 84.81%(67/79)]. mNGS had a receiver operating characteristic (ROC) curve of 0.775 for infectious disease, which was significantly higher than that of culture (0.661, $P < 0.05$). mNGS detection revealed that bacteria were the most commonly identified potential pathogens. The top causative pathogens identified were *Acinetobacter baumannii*. Of the 263 patients with FUO, clinical management of 48.67% (128/263) patients was positively affected by mNGS, and 51.33% (135/263) patients were not affected by mNGS($P = 0.1074$). To sum up, infectious diseases are the principal cause of FUO. mNGS could significantly improve the detected pathogen spectrum of FUO caused by infection. However, the FUO disease spectrum is relatively broad, including a large number of non-infectious diseases. Therefore, Further investigation is warranted into the specific clinical scenarios for which mNGS may offer the greatest clinical diagnostic value.

Keywords Metagenomic next-generation sequencing, Fever of unknown origin, Infection, Sensitivity, Specificity

Since 1961, Petersdorf and Beeson defined fever of unknown origin (FUO) as a temperature of 38.3 °C or higher for at least three weeks without identifiable cause despite at least a 1-week inpatient evaluation[1]. FUO is a challenging disease state for which potential underlying etiology can include infections, cancers, non-infectious inflammatory diseases, and miscellaneous causes[2]. The relative prevalence of various etiological subgroups of FUO differs across the globe[3]. Infection is the leading cause of FUO in China. Tuberculosis is one of the most common pathogens causing FUO[4]. Large shifts in the pathogens of FUO caused by the infection have occurred. The current era has witnessed a reduction in tuberculosis causes of FUO, with a rise in other bacterial infections (e.g., respiratory tract infections) and deep-seated infections (e.g., brain abscesses), which remain time-honoured entities associated with FUO[5]. The complex etiology poses challenges to the clinical diagnosis of FUO. Some studies show that undiagnosed cases account for 15–20% of cases[6]. The minimal initial diagnostic

[1]Department of Clinical laboratory, The First Affiliated Hospital, Jiangxi Medical College, Nanchang University, No.17, YongWaiZheng Street, Nanchang 330006, China. [2]Lan Min Lai, Qing-gen Chen and Yang Liu contributed equally to this work. ✉email: ndyfy06655@ncu.edu.cn

process of FUO includes detailed medical history, physical examination, basic laboratory tests (e.g., ESR and CRP), microbial cultures, serologic tests and imaging examinations[7]. If a final diagnosis cannot be obtained, additional testing (e.g., serologic or PCR testing for infectious pathogens or biopsy testing for other lesions) should be performed to discontinue new and potentially inappropriate medications[7]. At present, a considerable number of FUO patients still have unknown causes after undergoing systematic examinations[6]. Empirically administering antimicrobial or anti-inflammatory therapy in a patient with protracted fevers and unclear diagnosis is often tempting. However, therapeutic antimicrobial trials may predispose to resistance or suppress the growth of fastidious pathogens, potentially leading to the underlying cause of fever remaining untreated[8]. Identifying the pathogens responsible for infection-caused FUO remains a diagnostic dilemma, complicating the provision of early and effective treatment for physicians[9]. An optimized screening and diagnostic method is required for detecting pathogens of infection-caused FUO.

Traditional microbiologic testing is limited by antibiotic exposure, timing of testing, and reduced sensitivity[10]. Pathogen-specific polymerase chain reaction (PCR) assays require clinical doctors to have prior knowledge. Due to strict cultivation conditions, detecting newly emerging pathogens, viruses, and rare pathogens is often tricky[11]. Metagenomic next-generation sequencing (mNGS) technology is a high-throughput sequencing technique that involves unbiased sequencing of all genetic material in a specimen to obtain pathogen information and provide ancillary genomic information about antibiotic resistance genes (ARGs)[12,13]. To date, many studies have demonstrated its advantages in diagnosing infectious pathogens and minimizing the time of empirical treatment[14]. Moreover, mNGS has been reported to rule out infections and identify underlying etiology[15]. Currently, the research on mNGS in FUO is limited. This study aims to comprehensively evaluate the value of mNGS in the etiologic diagnosis of FUO by the pathogen spectrum and diagnostic performance, further clarifying the value of mNGS in clinical management.

Materials and methods

Study design

We retrospectively reviewed 281 patients with fever of unknown origin at the First Affiliated Hospital of Nanchang University from December 2020 to February 2023. The diagnostic criteria for FUO were defined as follows[1]: a group of diseases that cannot be diagnosed after one week of comprehensive physical examination in the outpatient or hospitalization; Fever lasts over three weeks, and oral temperature is > 38.3 °C at least three times (or at least three times the temperature fluctuates > 1.2 °C within one day. All enrolled FUO patients underwent mNGS and conventional cultures simultaneously. A total of 263 patients were included in the current study according to our inclusion/exclusion criteria (Fig. 1). The etiological diagnosis of FUO was made by experienced physicians, infectious experts and clinical microbiology experts, combining clinical manifestations, laboratory examination results, imaging results, treatment response, traditional test results, and mNGS results.

Metagenomic next-generation sequencing and analysis

All samples were collected according to standard procedures and stored at -80 °C. According to the manufacturer's manual, each sample was used for DNA extraction using the TIANamp Micro DNA Kit (TIANGEN Biotech, Beijing, China). Libraries were constructed by using the Nextera XT kit (Illumina). The Qubit (Thermo Fisher Scientific, MA, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA) were also utilized for library quality control. The NextSeq-550Dx sequencer was used for the sequencing reaction to acquire the sample sequence information. For bioinformatics analysis, adapter contamination, low-quality, and low-complexity reads were filtered by fastp (v0.19.5) and Komplexity (v0.3.62). Human host DNA reads mapping to the human reference assembly GRCh38 were removed with Bowtie2 (v2.3.4.3). The residual sequencing data were mapped to the Microbial Genome Databases. The supplementary material provides more detailed information on the mNGS method procedure. The positive detection criteria for mNGS are as follows[16]:

(1) For the detected bacteria (Mycobacterium excluded) and fungi (Cryptococcus excluded), the positive criteria for the mNGS result were set as follows: (a) The specifically mapped read number (SMRN) of each microbial taxonomy ranked top10 of the same kind of microbes and the microorganism was not detected in the NTC; or (b)Standardized SMRN(SDSMRN) > 1 (SDSMRN = SMRN×20 million/total reads).
(2) For the detected viruses, the positive criteria for the mNGS result were set as follows: (a) SDSMRN ranked top 3, and the microorganism was not detected in the NTC; and (b) SDSMRN > 5.
(3) Because of the low yield of DNA extraction and low possibility of contamination, pathogens such as parasites, *Mycobacterium spp*., *Rickettsia spp*., were considered identified when SDSMRN > 1, or it positioned first within the top 20 genus with the highest SDSMRN.

Diagnostic assessment of mNGS

Each mNGS result was identified as a true positive, false positive, true negative, or false negative result relative to the final clinical diagnosis. The diagnostic performance of mNGS and culture for the final clinical diagnosis was evaluated by using sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). To evaluate the correctness of the microorganisms detected by mNGS as the microorganism causal of the FUO and provide additional clarity regarding the criteria used in this study to define "True positive" and "False positive," This table outlines some general ideas to help clinical doctors to interpret the mNGS results in a useful way (Supplemental Table 1).

Statistical analysis

We used SPSS 26.0 and GraphPad Prism 9 for all statistical analyses. Counting data were presented as frequency and component ratio. Continuous variables with normal distribution were expressed as means ± SD, while non-

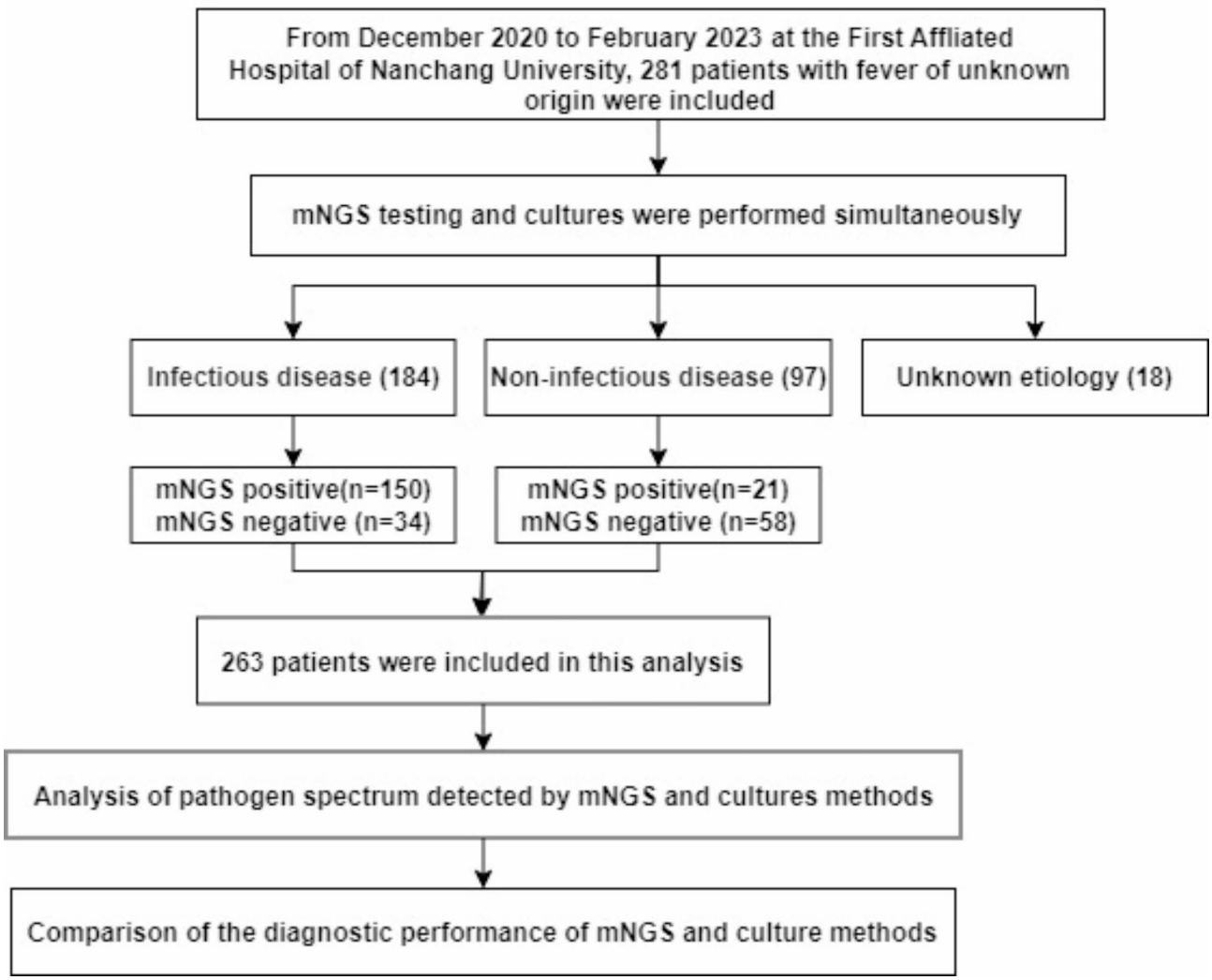


Fig. 1. Flowchart of study participants.

normal distribution variables were expressed as medians (25th, 75th percentiles). We compared continuous variables using the Mann-Whitney U-test or t-test and analyzed categorical variables using Fisher's exact test. A *P*-value of < 0.05 was considered statistically significant.

Results

Clinical characteristics and etiology of FUO

A total of 263 FUO participants, with a median age of 56.0 years (IQR 46–68 years), were retrospectively enrolled, of whom 173 (65.8%) were male. Hypertension is the most common co-morbidities (66/263, 25.1%). The clinical characteristics and laboratory examination are reported in Table 1. 78.7% (207/263) of patients had a history of empirical treatment before sampling. According to the final diagnosis, 184 patients were classified into the group of infectious disease (ID). Seventy-nine patients were categorized into the non-infectious disease (NID). Among infected patients who underwent or did not undergo empirical treatment before sampling, the detection rates of mNGS for potential pathogens were 83.67%(123/147) and 72.97%(27/37), respectively($P = 0.1339$). The inflammatory indicator CRP and average age in the ID group were significantly higher than in non-infectious disease groups ($P < 0.05$). The etiologic distribution of FUO was infections (69.96%), malignancies (9.51%), autoimmune/inflammatory diseases (7.22%), and miscellaneous diseases (13.31%). The most common infectious diseases were lower respiratory tract infections (53.3%), and the most common non-infectious diseases were tumors (16.5%).

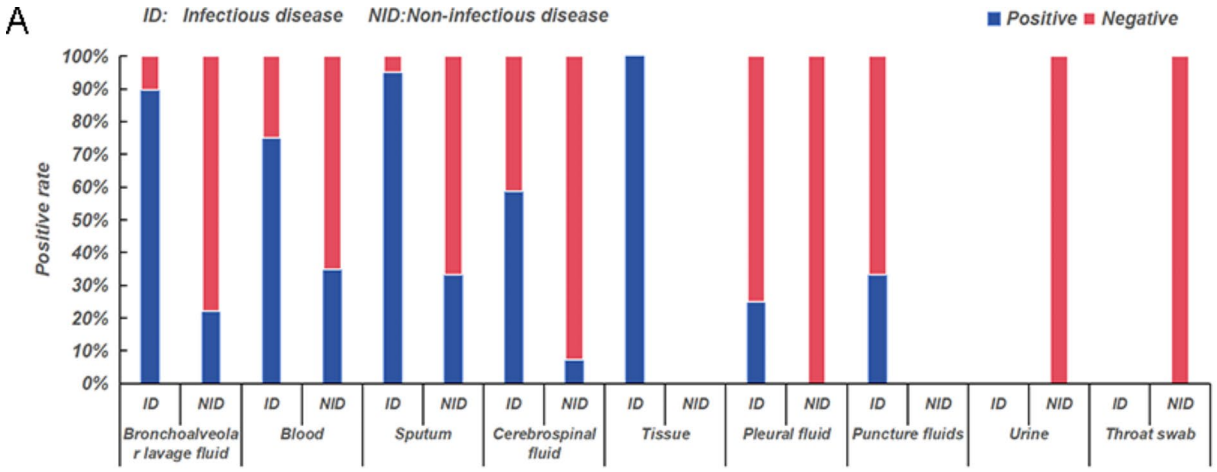
Comparison of mNGS and culture for pathogen detection

The sample types included bronchoalveolar lavage fluid(33.08%, 87/263), blood (40.30%, 106/263), sputum (9.89%, 26/263), cerebrospinal fluids (11.79%, 31/263), tissue(0.76%, 2/263), pleural fluid (2.28%, 6/263), throat swabs (0.38%, 1/263), urine (0.38%, 1/263), and puncture fluids(1.14%, 3/263). In infectious diseases, BALF(75.0%, 45/60) and sputum samples(95.0%, 19/20) are more positive than blood samples(89/98 vs.

	Overall (N = 263)	Infection(N = 184)	Non-infection (N = 79)	p value
Demographics				
Male/female	173/90	130/54	43/36	**0.0110**
Age(years), Median (IQR)	56.00(46.00, 68.00)	56.91(48.0, 69.00)	51.24(38.50, 61.50)	**0.0083**
Length of stay (days)	21.00(13.00, 33.00)	25.93(13.00, 31.75)	30.11(14.00, 40.50)	0.5077
Empirical treatment history	207	147(79.89)	60(75.95)	0.4741
Laboratory examination , Median (IQR)				
WBC, *10^9 /L	9.32(6.03, 14.67)	10.98(5.95, 13.63)	12.58(6.41, 17.36)	0.1368
Neutrophil,%	84.55(74.60, 90.78)	81.38(74.50, 91.10)	79.63(74.75, 89.28)	0.3659
Lymphocyte,%	8.45(4.33, 15.58)	12.78(4.20,15.70)	11.98(4.90, 15.08)	0.7323
CRP (normal 0–8 mg/L)	80.91(22.60,154.25)	106.92(33.00,161.53)	75.80(10.78, 109.61)	**0.0088**
ESR (normal 0–20)	42.00(18.50, 77.50)	50.00(22.75, 75.25)	48.76(8.00, 85.00)	0.8624
PCT (normal 0–0.5 ng/mL)	0.99(0.27, 5.25)	7.75(0.32, 4.68)	9.23(0.24, 5.25)	0.6158
Comorbidity (n)				
Hypertension	66	43	23	0.3246
Diabetes	45	29	16	0.3752
Cerebral infarction	15	12	3	0.3825
Surgical history	17	13	4	0.5450
Chronic kidney diseases	9	7	2	0.6027
Hepatitis B	2	2	0	0.3523
Etiology distribution of 263 FUO cases				
Infection disease(N = 184)	**No.**	**Non-infection disease (N = 79)**	**No.**	
Lower respiratory tract infection	98	Adult-onset Still's disease	5	
Bloodstream infection	50	Connective tissue disease	4	
Central nervous system infection	22	Systemic lupus erythematosus	2	
Intra-abdominal infection	9	Rheumatoid arthritis	1	
Skin or soft-tissue infection	1	Polymyalgia rheumatica	2	
Urinary tract infection	1	Hemophagocytic lymph histiocytosis	1	
Endocarditis	1	Multiple myeloma	1	
Tuberculous pleurisy	2	Myelodysplastic syndrome	2	
		Acute lymphoblastic leukemia	2	
		Acute myeloid leukemia	3	
		Lymphoma	6	
		Crohn disease	1	
		Dermatomyositis	1	
		Tumor	13	
		Others	35	

Table 1. Characteristics of patients with fever of unknown origin. Significant values are in bold. *mNGS* metagenomic next-generation sequencing, *WBC* white blood cell, *CRP* C-reactive protein, *ESR* erythrocyte sedimentation rate, *PCT* procalcitonin. Multi-site infection is counted by primary infection.

54/98)(Fig. 2A). To assess the diagnostic efficacy of mNGS, the Pathogens Extra Detection Rate (PEDR) of mNGS was calculated ($\mathrm{PEDR}_{\mathrm{mNGS/culture}} = \frac{\text{true mNGS positive cases} - \text{culture positive cases}}{\text{clinical definate infectious cases}}$) (Table 2). The overall $\mathrm{PEDR}_{\mathrm{mNGS/culture}}$ of infection was 28.8%. $\mathrm{PEDR}_{\mathrm{mNGS/culture}}$ of lower respiratory tract infection was 35.7% (89/98 vs. 54/98). $\mathrm{PEDR}_{\mathrm{mNGS/culture}}$ of bloodstream infection was 14.0%. $\mathrm{PEDR}_{\mathrm{mNGS/culture}}$ of central nervous system infection was 50.0%. However, the $\mathrm{PEDR}_{\mathrm{mNGS/culture}}$ of non-infection disease is also higher than in culture(11.4%; 21/79 vs. 12/79). We further analyzed the consistency between mNGS and the culture method. In both mNGS and culture, 85 of 184 cases tested positive for infection-caused FUO, and 22 of 184 cases were negative. Sixty-five cases were mNGS test positive and culture negative. 12 cases had only culture-positive results(Table 2). Taking final clinical diagnosis as the gold standard, Each mNGS result was identified as a true positive (TP), false positive (FP), true negative (TN), or false negative (FN) result, and the sensitivity and



B

Clinical Diagnosis

mNGS	Pos	Neg
Pos	150	21
Neg	34	58

Sensitivity (95% CI)=81.5% [75.3-86.5]
Specificity(95% CI)=73.4% [62.8-81.9]
PPV (95% CI) =87.7% [82.0-91.8]
NPV (95% CI=63.0% [52.8-72.2]

Clinical Diagnosis

Culture	Pos	Neg
Pos	87	12
Neg	97	67

Sensitivity (95% CI)=47.3% [40.2-54.5]
Specificity(95% CI)=84.8% [75.3-91.1]
PPV (95% CI) =87.9% [80.0-92.9]
NPV (95% CI=40.9% [33.6-48.5]

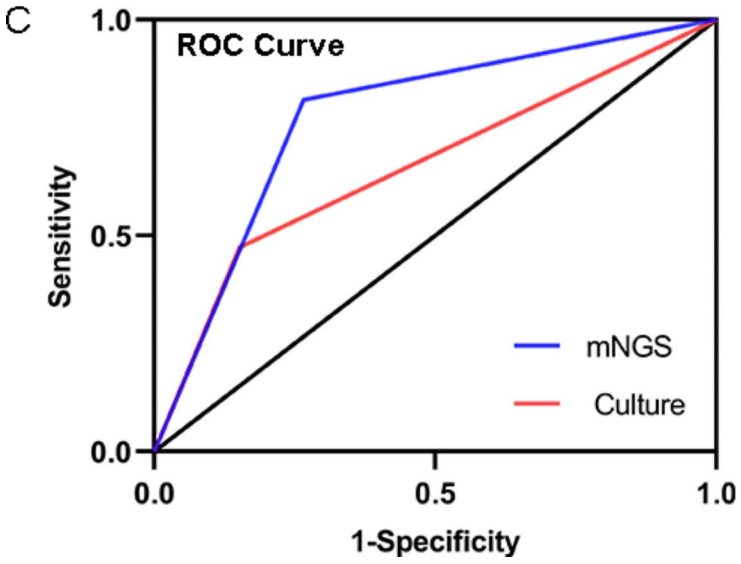


Fig. 2. (**A**) Distribution of the positive rates of different sample types; (**B**) The diagnostic efficiency of mNGS and cultures based on the clinical diagnosis; (**C**) Comparison of the diagnostic efficacy of mNGS and culture methods in infection group using ROC curves. *PPV* positive predictive value, *NPV* negative predictive value.

specificity were calculated to evaluate the diagnostic value of mNGS. We calculated mNGS sensitivity compared to clinical diagnosis=mNGS-positive/clinically diagnosed infections. The specificity compared to clinical diagnosis = mNGS-negative/clinically diagnosed non-infections. The supplementary material shows a more detailed definition and calculation formula of sensitivity and specificity. From these cases, mNGS identified 150 true-positive cases, 21 false-positive cases, 58 true-negative cases, and 34 false-negative cases. We found

	No.	NGS(+)/ culture(+)	NGS(+)/ culture(−)	NGS(−)/ culture(+)	NGS(−)/ culture(−)	$PEDR_{mNGS/culture}$
Infectious disease	184	85	65	12	22	28.8%
Lower respiratory tract infection	98	50	39	4	5	35.7%
Bloodstream infection	50	27	13	6	4	14.0%
Intra-abdominal infection	9	3	1	2	3	-11.1%
Central nervous system infection	22	4	11	0	7	50.0%
Skin or soft-tissue infections	1	0	0	0	1	0.0%
Urinary tract infection	1	0	0	0	1	0.0%
Endocarditis	1	0	1	0	0	100.0%
Tuberculous pleurisy	2	1	0	0	1	0.0%
Non-infection disease	79	6	15	6	52	11.4%

Table 2. The diagnostic value of mNGS compared to culture in FUO. *PEDR* Pathogens Extra Detection Rate, $\mathrm{PEDR}_{\mathrm{mNGS/culture}} = \frac{\text{true mNGS positive cases} - \text{culture positive cases}}{\text{clinical definate infectious cases}}$.

that mNGS showed higher sensitivity compared with culture (81.5% vs. 47.3%), although the specificity of mNGS testing was lower than that of culture tests (73.4% vs. 84.8%)(Fig. 2B). mNGS had a receiver operating characteristic (ROC) curve of 0.775 for infectious disease, which was significantly higher than that of culture (0.661, $P<0.05$) (Fig. 2C).

Pathogen distribution detected by mNGS and culture methods

We detected 303 suspected pathogens in the sample of 263 patients using mNGS. Overall, mNGS detected an additional 23 types of bacteria and fungi in culture-negative patients(Fig. 3A). Of the 303 microorganisms, Gram-negative bacilli (GNB) were the most common pathogens(38.3%,116/303), followed by viruses(32.0%,97/303). Viruses accounted for the highest proportion of sequences (25.3%, 20/79) among non-infectious diseases, possibly related to *Human cytomegalovirus*, *Epstein-Barr virus*, or *Herpes simplex virus type 1* latency (Fig. 3A). 184 patients were diagnosed with infectious diseases, including 59 cases of bacterial infection, 22 cases of viral infection, 9 cases of fungal infection, 7 cases of Mycoplasma/Chlamydia/Rickettsia infection, and 1 case of parasitic infection. In addition, 52 mixed infection cases were detected. Among them, combined bacterial and viral infections accounted for 55.8% (29/52)(Fig. 3B). The results of pathogens detected by mNGS showed that the bacteria was the most prevalent pathogen, in which the most frequently detected were *Acinetobacter baumannii* and then *Klebsiella pneumoniae*. The top identified fungi were *Candida albicans* and *Pneumocystis jirovecii*. The top identified viruses were CMV and HSV-1*1*(Fig. 3A).

The application of mNGS in assisting clinical care

Of the 263 patients with FUO, 48.67% (128/263) of clinical management was positively affected by mNGS. 51.33% (135/263) of patients were not affected by mNGS($P=0.1074$)(Table 3). Out of 171 mNGS-positive patients, clinical management was positively affected for 45.03%(77/171) patients, such as identifying new pathogens and changing antibiotic regimens. For 54.9% (94/171) patients, their treatment regimen remained unchanged due to the unknown significance of pathogen detection or the previous antibiotic regimen covering the pathogen. Additionally, 51 patients received modified clinical management based on negative mNGS test results, leading to the de-escalation of antibiotics and avoidance of invasive tests such as tissue biopsy, based on negative mNGS test results suggesting a low likelihood of pathogen infection.

Discussion

The clinical manifestations of FUO are diverse and have complex etiologies[2]. A previous study investigated the distribution of causes of FUO in China between 2013 and 2022 and found that infection remains the leading cause of FUO, which facilitates the clinical understanding of the etiology of FUO[4]. Similar to this study, infection was the main causative factor in etiologic classification, accounting for 69.9% of various etiologies in our research. The complex and diverse pathogens pose challenges to the diagnosis and treatment decisions of infection-caused FUO. Therefore, obtaining accurate pathogen detection results is crucial for diagnosing patients with FUO caused by infection. mNGS holds promise in diagnosing FUO by detecting a wide range of pathogens. However, relatively few evaluations have been conducted on the etiological diagnostic value of mNGS in FUO.

In this study, we conducted a retrospective cohort study to assess the diagnostic utility of mNGS techniques in 263 FUO patients. mNGS provides a greater coverage of pathogen detection than culture methods. The overall pathogens extra detection rate (PEDR) of mNGS has a 28.8% improvement compared to culture in infection-caused FUO. Identifying potential pathogens is often crucial in the most severe cases, such as meningitis or infections in cancer patients or immune-compromised patients due to different subjacent pathologies. In addition, the proportion of mixed infections (28.3%, 52/184) in our patients was much higher than we originally thought. Previous studies have demonstrated that mNGS is notably superior to conventional tests such as PCR or culture in identifying coinfections[17]. In this study, mNGS have demonstrated the ability to identify slow-growing and fastidious bacteria(e.g., *Mycobacterium tuberculosis* and *toxoplasma*) from culture-negative specimens, providing support to the idea that mNGS is a quicker and more effective approach for identifying rare or uncommon pathogens[18,19]. mNGS also has a significant advantage over culture methods in terms of

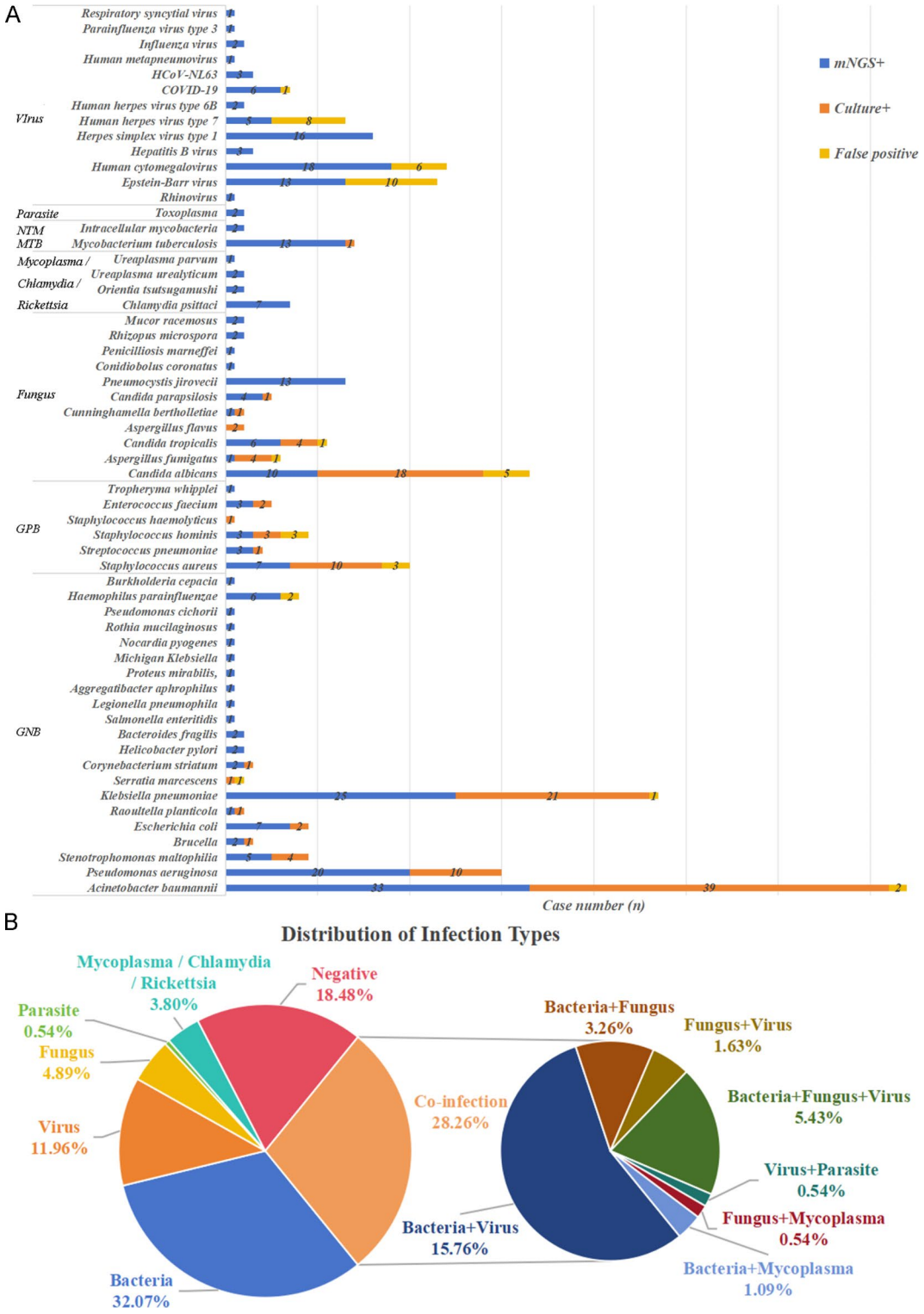


Fig. 3. (**A**) Distribution of pathogens identified by mNGS and Cultures; (**B**) Distribution of infection types detected by mNGS.

NPV. This result implies that mNGS may also be suitable for ruling out infections, thereby focusing the doctor's attention on non-infection-associated FUO[20].

Our findings indicate that mNGS demonstrates higher sensitivity and lower specificity in identifying the etiology of FUO patients compared to culture methods. mNGS detected additional pathogens in 21 patients diagnosed with non-infections in our research. The most common pathogens were viruses (95.2%, 20/21), possibly related to CMV, EBV, or HSV-1 latency. There is still no uniform standard to determine whether the

mNGS result	Change in management	Clinical impact[1]	Patient($n=263$), count(%)	*P* value
Positive	Yes	New diagnosis based on mNGS result and not conformed by CMTs[4], mNGS result enabled initiation of targeted antimicrobial therapy	46 (17.49)	0.1074
		Earlier diagnosis based on mNGS result, later confrmed by CMTs, mNGS result enabled initiation of targeted antimicrobial therapy	31 (11.79)	
Positive	No[2]	mNGS result showed new organism, but antibiotics and clinical plan were not changed	28 (10.65)	
		mNGS result confirmed CMTs diagnosis and no additional action	66 (25.10)	
Negative[3]	Yes	No mNGS result confirmed clinical diagnosis, de-escalated treatment of antimicrobial	38 (14.45)	
		No mNGS result confirmed clinical diagnosis, avoid invasive surgical biopsy	13 (4.94)	
Negative	No	mNGS test result was negative, no action taken	41 (15.59)	

Table 3. Clinical impact of mNGS by patient with fever of unknown origin. (1) Clinical impact were defined by a composite reference method, interpreting clinical history and all microbiological data, including mNGS findings; (2) Non-replacement due to antimicrobial coverage of the current pathogen was included in this item; (3) If both de-escalated treatment of antimicrobial and avoid invasive surgical biopsy are present, only the primary one will be recorded. 4.CMTs: conventional microbiological tests, cultures were performed simultaneously, other conventional microbiological methods, such as culture, serologic tests, PCR were performed according to the clinical necessity.

viruses detected in blood were pathogenic or virus-carrying[21]. In practice, the clinical significance of the virus needs to be determined based on the patient's medical history and clinical symptoms. Other false positives likely result from contamination in sample collection, colonizing bacteria, disrupted mucosal barrier function, and library preparation[22,23]. Due to the lack of unified standards for interpreting mNGS results, clinicians must make a comprehensive judgment when identifying pathogenic bacteria. Therefore, it is still important to remember that "true detection" does not necessarily mean "true infection."

Research found that the proportion of infection-caused FUO patients attributed to respiratory tract infections and bloodstream infections has increased recently[24]. In our study, The most common type of infectious disease was lower respiratory tract infection, consistent with a meta-analysis of 16,278 patients with FUO in China[4]. The positivity rate of mNGS varies among different types of specimens. BALF and sputum samples are more positive in infectious diseases than blood samples. The high level of host DNA in blood samples can limit pathogen sequence yield, reducing the sensitivity of blood mNGS analysis[25]. In addition, most samples sent to examine focal infections are peripheral blood, which may also limit the positivity rate of blood mNGS[26]. Fu et al. proposed that collecting specimens from suspected primary infection sites can improve the accuracy of pathogen detection[16].

Large shifts in the pathogenic bacteria of infection-caused FUO have occurred during the past century[7]. The rates attributed to tuberculosis have significantly decreased in recent years compared with those in the previous decade[4]. Hospital-acquired pneumonia (HAP) caused by opportunistic pathogens is related to the increase in the incidence rate of FUO[27]. *Acinetobacter baumannii*, the cause of hospital-acquired pneumonia (HAP), is the most common microorganism in sputum cultures of long-term hospitalized patients[28]. This may explain why the most frequent pathogen detected by mNGS is *Acinetobacter baumannii*. However, the *Acinetobacter baumannii*, *Candida albicans*, and *Staphylococcus aureus* positivity rates detected by mNGS were notably lower than that identified by culture, potentially due to the absence of these pathogen sequences in the top 10 organisms list or the stringent threshold settings or insufficient sequencing depth[29]. In addition, a potential reason for failing to detect the specific microorganism responsible for the infection through mNGS could be an incorrect selection of sample type[30].

Studies have differing views about mNGS' impact on clinical outcomes, which can be attributed to differences in patient populations, study designs, and definitions of what constitutes clinical impact. The Children's Hospital of Chicago found that 56% of the mNGS samples provided clinically relevant results and changes in clinical treatment decisions[31]. In a retrospective chart review of 142 mNGS test results from a tertiary pediatric hospital, Wilke et al. found that 32.4% of the results directly influenced changes in clinical management[32]. In contrast, Niles et al. found little benefit from ordering mNGS concurrently with routine testing[33]. Other studies have demonstrated that mNGS has positive effects primarily because of the de-escalation of antimicrobial therapy[34]. Many patients have empirically selected antimicrobials that already cover current pathogens in our research. Therefore, changes in clinical management rates were lower than mNGS-proven infection rates.

mNGS provides more excellent pathogen detection coverage than traditional testing and has a specific value for diagnosing pathogen infections that are difficult to culture routinely. However, mNGS testing is highly dependent on the sequencing depth; a high content of host DNA in samples may limit the yield of pathogen sequences, thus reducing the sensitivity of mNGS detection[23]. In addition, mNGS cannot distinguish pathogenic microorganisms from colonizing microorganisms, background microorganisms, and contaminated microorganisms, which remains a great challenge for clinicians[35]. It is sometimes necessary to validate mNGS results using other techniques to address this issue. In the future, we believe that assembling an interdisciplinary team, which includes medical microbiologists, computational biologists, epidemiologists, infectious disease specialists, and other clinicians, along with targeted training, is crucial for the optimal utilization of mNGS.

There were some limitations in our study. Firstly, this was a single-centre retrospective study, and the composition of patients with FUO and the sample might be biased. Secondly, our results did not cover FUO

patients who did not undergo mNGS testing. There was bias in the enrollment of FUO patients. Future prospective studies on a larger scale will be beneficial for further verifying the diagnostic and therapeutic value of mNGS for FUO etiology under investigation.

Conclusion

Infections represent the most frequent category among final diagnosis of FUO. Using mNGS to detect pathogens in FUO holds higher diagnostic sensitivity than culture, which is conducive to clarifying the etiology of infection-caused FUO patients. However, the FUO disease spectrum is relatively broad, including a large number of non-infectious diseases. Signs of infection and effective selection of samples may facilitate better utilization of mNGS' unbiased characteristics, ultimately leading to a significant improvement in FUO patients' diagnostic rate. Further investigation is warranted into the specific clinical scenarios for which mNGS may offer the greatest clinical diagnostic value.

Data availability

All data generated or analyzed during this study are included in this published article. The data of this study are available from the corresponding author on reasonable request.

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Declarations

Competing interests
The authors declare no competing interests.

Ethics approval and consent to participate
This study was approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University(No. IIT-2024-386). As this is a retrospective study, the Ethics Committee of the First Affiliated Hospital of Nanchang University granted the study exemption status of informed consent. In addition, we declare that this study is in line with the ethical guidelines of the Declaration of Helsinki, and the patient-related data is strictly confidential. All methods were carried out per the manuscript's relevant guidelines and regulations.

Additional information
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Correspondence and requests for materials should be addressed to L.Y.