

# Incremental value of metagenomic next generation sequencing for the diagnosis of suspected focal infection in adults

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## SUMMARY

**Objectives:** Microbiological diagnosis is essential during clinical management of focal infections. Metagenomic next generation sequencing (mNGS) has been reported as a promising diagnostic tool in infectious diseases. However, little is known about the clinical utility of mNGS in focal infections.

**Methods:** We conducted a single-center retrospective study to investigate impact of mNGS on focal infection diagnosis and compared it with conventional methods, including culture, pathological examination, Xpert MTB/RIF, etc. 98 suspected focal infections cases were enrolled, and medical records were reviewed to determine their rates of detection, time-to-identification, and clinical outcomes.

**Results:** mNGS showed a satisfying diagnostic positive percent agreement of 86.30% (95% CI: 75.79–92.88%) in a variety of tissues, compared to 45.21% (95% CI: 33.68–57.24%) for culture and 57.53% (95% CI: 45.43–68.84%) for conventional methods ( $p < 0.0125$ ), and detected an extra 34 pathogenic microorganisms. Time requirement for pathogen identification using mNGS ranges from 31 h to 55 h, which showed an advantage over culture. (82.36 h; 95%CI: 65.83, 98.89;  $P < 0.05$ )

**Conclusions:** mNGS showed promising potential in pathogenic diagnosis during focal infections and might enable clinicians to make more timely and targeted therapeutic decisions.

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## Introduction

Focal infection is a cluster of diseases during which infections were localized in a specific part of organs and can occur in virtually every organ of a human body, such as eyes, bones, etc.<sup>1–4</sup> Focal infection can be divided into two parts, purulent infections and non-purulent necrotizing tissue infections. Rapid and precise microbiological diagnosis is one of the keystones of the management of diseases to improve patient prognosis. The golden standard for bacterial pathogen diagnosis is undoubtedly culture, but weakness of which still exists in that fastidious bacteria culture is relatively difficult and the culture time con-

sumption fluctuated among different pathogens. Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF)<sup>5,6</sup> has been shown to further increase positive percent agreement through direct identification of pathogens from samples like urine, but it is still more widely used in identifying pathogens from culture-positive specimens.<sup>7,8</sup> Multiplex PCR assays (Filmarray, etc.)<sup>9–12</sup> usually could only detect a limited range of microorganisms. Therefore, new techniques, which had the capability to overcome limitations of current diagnostic tests and allows for advantages of hypothesis-free, culture-independent, and direct pathogen detection from clinical specimens, will further assist clinicians' approaches to focal infection.

Hypothesis-free testing metagenomics next generation sequencing (mNGS) offers a relatively unprejudiced and unlimited diagnostic tool for all microorganisms whose sequencing data was included in database library. Up till now, multiple articles have highlighted its value in pathogen detection from blood,<sup>13–15</sup> respiratory tract,<sup>16,17</sup> cerebral spinal fluid samples<sup>18–20</sup> and prosthetic joint infection.<sup>21,22</sup> However, data of the diagnostic efficacy of mNGS still lacks in the field of focal infection in all parts of the body, and this cohort study aimed to establish an mNGS analy-

**Abbreviations:** MALDI-TOF, Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry; mNGS, metagenomic Next generation sequencing; PCR, specific polymerase chain reaction; OR, odd ratio; 95% CI, 95%: confidence intervals; MTB complex, Mycobacterium tuberculosis complex; SSTI, skin and soft tissue infections; USA, The United States of America; DNA, deoxyribonucleic acid; RNA, ribonucleic acid.

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**Table 1**  
Criteria of focal infection.

<p><b>A: Criteria of suspected focal infection</b> One of the following two criteria should be met: 1. If the infection lesion is in skin or visible soft tissue, at least one of the following criteria should be met: a. Redness, swelling, painful, or fluctuate in focal lesion b. Drainage of pyogenic fluids from the sinus tract 2. If the visible focal lesion is detected by imagological examination or other invasive procedure, at least one of the following criteria should be met: a. A history of injury, invasive procedures or immunosuppression b. Fever with a spike over 38.3 °C c. An elevated level of leukocyte count, PCT, CRP, ESR or serum ferritin that could not be explained by a non-infection reason.</p> <p><b>B: Criteria of clinical and pathogenic diagnosis of focal infection</b> 1. The clinical diagnosis was made by the following criteria: Patient's condition improves and the focal lesion reduces or disappears after treatment of targeted antimicrobial agents (two independent experienced clinicians analyzed the results and made the adjudication whether the results of mNGS results were in accordance with the clinical diagnosis). 2. Pathogenic diagnosis was made by the following method: a. There is a positive culture result of pyogenic fluids or tissue sample. b. There is a positive Xpert MTB/RIF result of pyogenic fluids or tissue sample DNA. c. There is a positive pathogen finding by pyogenic fluids smear, tissue pathology or skin imprint cytology d. There is a positive mNGS result confirmed by specific PCR. The positive criteria for mNGS is as follow: Step 1. Pathogens owning the highest <math>TA_i</math> in their genus. Step 2. Pathogens' <math>TA_i</math> ranks top 10 after Step 1. Step 3. After the previous 2 steps, pathogens whose <math>KA_i</math> exceed the threshold of the belonging kingdom (5%) and have a standardized unique reads (SDSMRN) &gt; 10 are selected.</p> <p><b>C: Criteria of final diagnosis of focal infection</b> 1. Definite focal infection: both pathogenic diagnosis and clinical diagnosis were met 2. Possible focal infection: only clinical diagnosis was met 3. Non-focal infection: none of criteria was met.</p>
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sis platform to evaluate its diagnostic value in localized infection further.

## Materials and methods

### Study design and participants

This single-center retrospective cross-section study was conducted in Huashan Hospital, Shanghai, China from March 2017 to December 2017. Patients, aged over 16, who met the diagnostic criteria of suspected focal infection (Table 1A) were consecutively enrolled. All of the collected samples were sent for culture and smear, and the tissue samples were also sent for pathology and imprint cytology. The physicians prescribed other conventional tests such as Xpert MTB/RIF and smear tests according to their clinical judgment of necessity.<sup>23</sup> Complete clinical microbiological data was available for all samples.

Written informed consent was obtained for each patient prior to participation.

Synchronous duplicate specimens were later sent for mNGS. The positive results of mNGS were followed by a specific polymerase chain reaction (PCR) validation to exclude the possibility of false positivity after mNGS.

Patients' electronic medical records were screened for baseline information, including age, sex, patients' immunosuppressive condition, highest temperature during disease course, whole blood cell count, erythrocyte sedimentation rate, C-reaction protein, serum ferritin, procalcitonin, specimen smear, culture and pathological results, and whether patients had received effective treatment prior to admission. We defined effective treatment as usage of antimicrobial therapy that was sensitive to pathogens later determined as the causative agents during focal infection, or that patients' symptoms were considered relieved after prescription of the medication (Fig. 1). Treatment length was calculated from the first day effective antimicrobial therapy had been prescribed. The study was approved by the ethics review committee of Huashan Hospital.

### Pyogenic fluids/tissue preparation and mNGS process

The pyogenic fluids/tissue samples were added with 0.5 mL BioSpec beads (0.5 mm dia. ZIRCONIA/SILICA Cat. No. 11079105z),

and shook intensely with the equipment of Mini-BeadBeater for 2 min in the agitation step before nucleic acid extraction. For mNGS, 0.5 mL of pyogenic fluids was put in a 1.5 mL microcentrifuge tube and were agitated vigorously at 2800–3200 rpm for 30 min to extract DNA. RNA was not sequenced in all of our specimens, and we only marked some of the samples which were suspected of virus infection by clinicians at the time of sample collection. DNA and, on a case-by-case basis, total RNA were extracted with TIANamp Micro DNA Kit (DP316, TIANGEN BIOTECH, Beijing, China) and QIAamp Viral RNA Mini Kit (52906, Qiagen, China) following the manufacturer's operational manual, respectively. The RNA was reverse transcribed and synthesized to double-stranded complementary DNA (ds cDNA) with SuperScript II Reverse Transcription Kit (18064-014, Invitrogen, China). After synthesis of second-strand DNA, DNA libraries were constructed through DNA-fragmentation, end-repair, add A-tailing, adapter-ligation and PCR amplification. Agilent 2100 was used for quality control and DNA libraries were then sequenced by BGISEQ-100 platform.

All raw reads were quality filtered using made-in-house program, including filtering adapter contamination, low quality and low-complexity reads. Next, the clean reads after quality filtering were mapped to a human reference database including hg19 and Yanhuang genome sequence using Burrows–Wheeler Alignment (Version: 0.7.10). Remained reads were aligned to the nonredundant bacterial, virus, fungal, and parasite databases using Burrows–Wheeler Alignment (Version: 0.7.10). The mapped data were processed for advanced data analysis. The genome databases were downloaded from NCBI (<ftp://ftp.ncbi.nlm.nih.gov/genomes/>). RefSeq contains 2700 whole genome sequences of viral taxa, 1494 bacterial genomes or scaffolds, 73 fungi and 47 parasites associated with human diseases (Supplementary material 1). We uploaded the raw data onto China national GeneBank (CNP0000607).

### Diagnosis of focal infections

The initial clinical diagnosis was performed by two independent experienced clinicians. After high-throughput sequencing is conducted, two other independent researchers interpreted and analyzed both mNGS results and its accordance with clinical manifestation. Final diagnosis of focal infection was made based

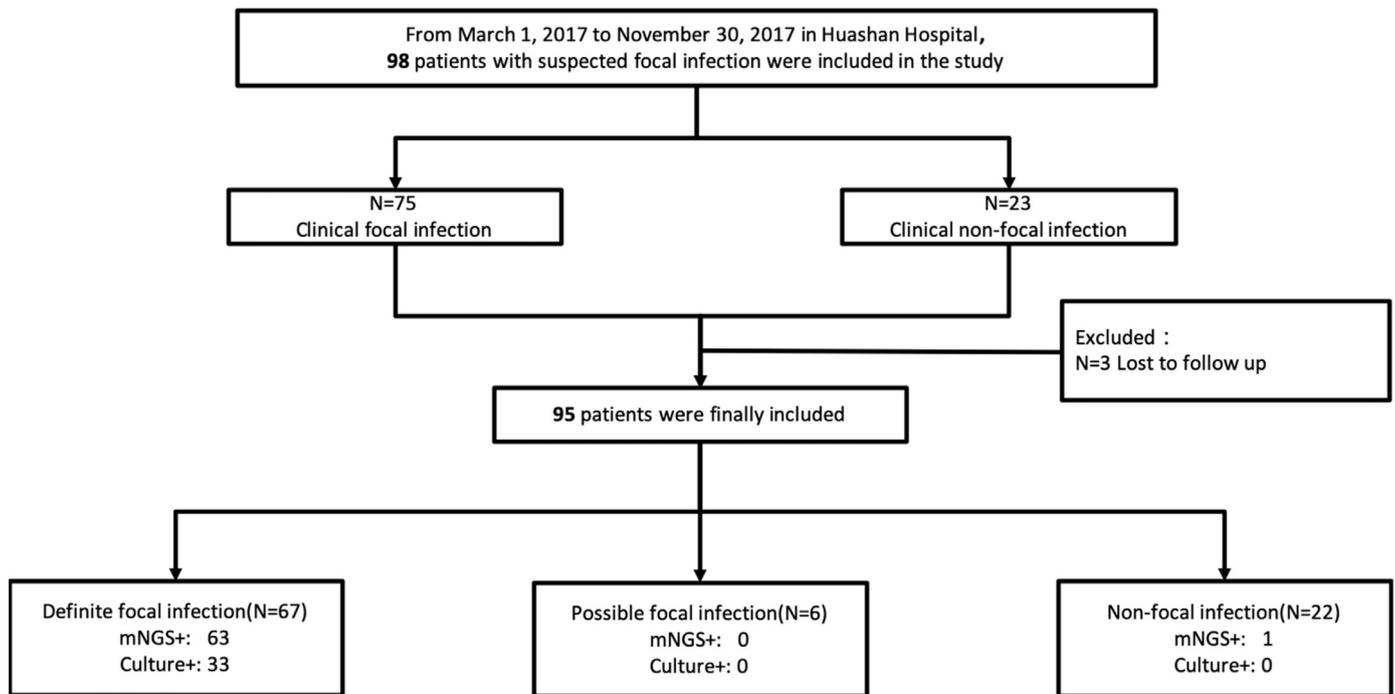


Fig. 1. Trial profile.

on combination of pathogen and clinical diagnosis (Table 1B and C), including definite focal infection, possible focal infection and non-focal infection.

The sequencing data of each sample is categorized into 4 tables each representing bacteria, fungi, virus and parasite, and one complete table was merged.

The absolute abundance ( $B_i$ ) of the specific pathogen can be calculated by:

$$B_i = \frac{X_i}{L_i}$$

Where  $X_i$  means the reads of the specific pathogen in the sample,  $L_i$  refers to the whole length of the pathogen genome.

The relative abundance ( $A_i$ ) of the specific pathogen can be calculated by:

$$A_i = \frac{B_j}{\sum_j B_j}$$

Two different types of relative abundance for each pathogen could be calculated.  $KA_i$  was defined as pathogen's relative abundance using data solely from its belonging kingdom list, while  $TA_i$  was calculated using the data from the total merged list (Table 1 and C).

#### Statistical analysis

Baseline characteristics were analyzed using the Mann–Whitney  $U$  test and the Chi-square test. Results were reported as adjusted odds ratios (OR) with their 95% confidence intervals (95% CI).  $P$  values  $< 0.05$  were considered statistically significant. During diagnostic value performance evaluation, data was corrected for multiple comparisons using Bonferroni (Dunn) method with adjusted  $p$  value and a 2-sided McNemar test was used to compare differences in diagnostic performance in all samples. Statistical analyses and figures were conducted using the SPSS statistical package 12.0 software and GraphPad Prism 5 software.

## Results

### General characteristics of the enrolled cohort

A total of 98 patients with suspected focal infection were consented for sample collection and went through screening. Three patients were lost to follow up, and three samples failed quality testing during deep sequencing. Finally, 95 patients were enrolled, among which, 67 patients were diagnosed as definite focal infection, six patients were diagnosed as possible focal infection, and 22 patients had non-infectious diseases including vasculitis, tumor, etc. (Fig. 1) (Supplementary material 2). Baseline characteristics (Supplementary material 3) demonstrated that no variables showed statistical difference between focal infection group (definite and possible) and non-infection group. Fourteen patients in the study had the condition of immunosuppression (Supplementary material 4). 23.29% of the patients had been effectively treated with antimicrobial therapy. Skin, muscle and bone infections were among the most common focal infections treated in our hospital while other parts of diseases were also enrolled (Fig. 2(A) and (B)).

### Diagnostic performance of mNGS in focal infection

The positive percent agreement of mNGS compared to clinical diagnosis was significantly higher than that of culture and conventional methods (86.30%; (95% CI: 75.79–92.88%) vs. 45.21%; (95% CI: 33.68–57.24%), 57.53%; (95% CI: 45.43–68.84%),  $p < 0.0125$ ) (Table 2A, Supplementary material 5), and combination of the above methods would further increase the positive percent agreement. Identical test between mNGS and culture was 90.91% and 88.89% in a culture-positive group and conventional methods-positive (excluding culture) group, respectively. mNGS detected an extra 34 pathogens compared with conventional methods and raised the diagnostic rate by 46.58% (34/73) (Table 2B). Four false negative and three false positive cases were reported (Supplementary material 6). In 31 cases of skin and soft tissue infections (SSTI), the positive percent agreement of mNGS and the combination methods were statistically higher than culture ( $p < 0.0025$ ).

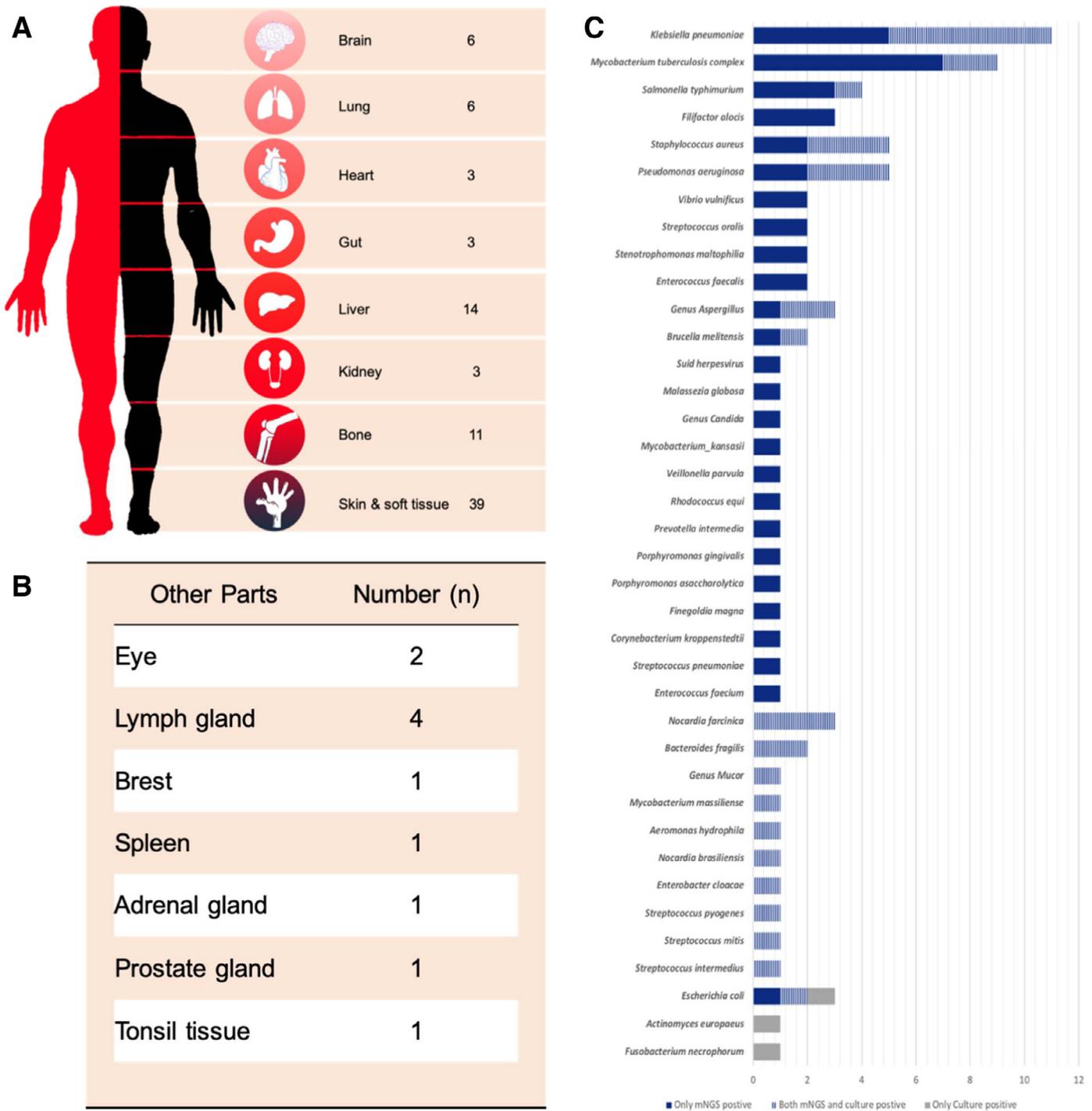


Fig. 2. (A, B) Distribution of collected tissue specimens. (C) Distribution of detected pathogens and identical performances for focal infections of mNGS and culture.

(Table 2A). A total of 16 cases had been effectively treated before admitted to our hospital. Comparing to non-effectively treated patients, the positive percent agreement of mNGS showed no significant decline while culture showed a trend of decreasing in effectively treated groups. Treatment length had no significant impact on positive percent agreement of both mNGS and culture in our study (Supplementary material 7A and 7B).

#### Distribution of identified pathogens

In this study, *Klebsiella pneumoniae* was the most detected pathogens, followed by *Mycobacterium tuberculosis complex*

(MTB complex) (Fig. 2(C)). mNGS rose superior to culture in identification of pathogens never reported to infect human (*Suid Herpesvirus*) and detection of causative agents that either had a relatively low culture rate or demanded time-consuming culture process, like *Vibrio vulnificus*, MTB complex, etc.

The majority of pathogen identified in purulent fluids and tissues reported a comparatively high amount of standard unique reads, leaving out *Brucella melitensis*, *Salmonella typhimurium*, Genus *Nocardia* and MTB complex, whose average standard unique reads were below 10 (Supplementary material 8).

Background sequences could be partitioned into two categories (Supplementary material 9), pathogens detected by mNGS but had

**Table 2**

Diagnostic value of mNGS in focal infection.

	Positive percent agreement	Negative percent agreement
<b>Total samples (n/N;95%CI)</b>		
Culture	45.21%(33/73; 33.68–57.24%)	100%(22/22;81.50–100%)
Conventional methods	57.53%(42/73; 45.43–68.84%)	100%(22/22;81.50–100%)
mNGS	86.30%(63/73; 75.79–92.88%) <sup>b</sup>	95.45%(21/22;75.12–99.76%)
Combination methods <sup>a</sup>	89.94%(65/73; 75.11–94.81%) <sup>b</sup>	95.45%(21/22;75.12–99.76%)
<b>Skin and soft tissue infection (SSTI) (n/N;95%CI)</b>		
Culture	54.83%(17/31;36.30–72.21%)	100%(8/8;59.77–100%)
Conventional methods	70.97%(22/31;51.76–85.11%)	100%(8/8;59.77–100%)
mNGS	90.32%(28/31; 73.10–97.47%) <sup>c</sup>	100%(8/8;59.77–100%)
Combination methods	93.55%(29/31; 77.16–98.87%) <sup>c</sup>	100%(8/8;59.77–100%)

**A: Diagnostic performance for focal infection of mNGS, conventional methods and combined methods**

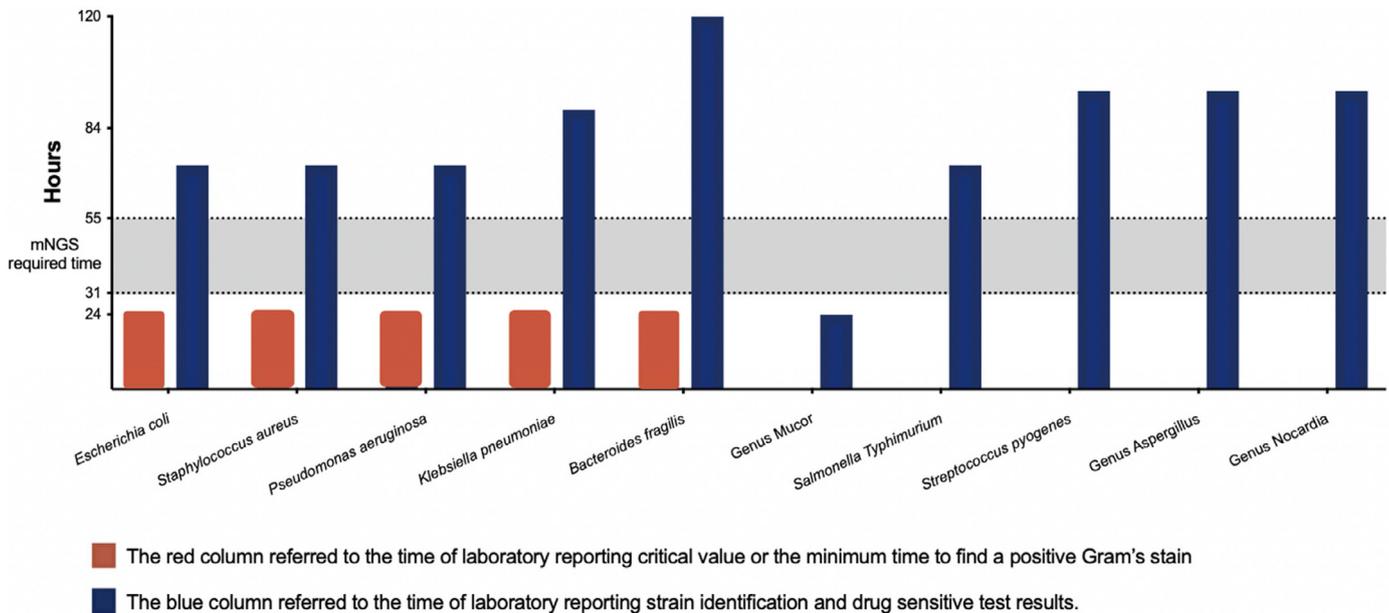
	Samples (n)	Identical findings (n, %)	Pathogens not identified by mNGS (n, %)	Extra pathogens identified by mNGS (n, %)
Non-focal infection	22	21 (95.45%)	N/A	1 (4.55%)
Culture-positive focal infection	33	30 (90.91%)	3 (9.09%)	0(0%)
Culture-negative focal infection	40	6 (15.00%)	N/A	34 (85.00%)
Conventional methods-positive (excluding culture) focal infection	9	8 (88.89%)	1 (11.11%)	0(0%)
Conventional methods-negative focal infection	1	0(0%)	0(0%)	1(100%)

**B: Identical and discrepant performance for focal infections of mNGS, culture and conventional methods**

<sup>a</sup> Combination methods: the combination of metagenomic next generation sequencing and conventional methods including: culture, pathology, Xpert MTB/RIF, smear tests, imprint cytology.

<sup>b</sup> The positive percent agreement of mNGS and combination methods is significantly higher than that of conventional methods and culture.

<sup>c</sup> The positive percent agreement of mNGS and combination methods is significantly higher than that of culture in skin and soft tissue infection.

**Fig. 3.** Time consumption comparison between mNGS and culture in focal infection.

no previous report of specific organ/tissue infections, and common pathogens colonized in this particular body region.

**Comparison of time consumption between mNGS and traditional smear/culture**

We compared pathogen-identification time consumption of both smear/culture (MALDI-TOF was excluded for not having been formally applied in our hospital) and mNGS whose turnaround time ranged from 31 h to 55 h (82.36 h; 95%CI: 65.83,98.89;  $P < 0.05$ ) (Fig. 3). Critical value reports by gram-staining were all within 24 h. However, it had a relatively low positivity and can hardly identify bacteria to the specific species. Average time of mNGS process took 31–55 h per sample and showed an advantage

over traditional culture in identification of the particular microorganisms.

**Discussion**

Here, we conducted a single-center retrospective cross-section study to evaluate the use of metagenomic shotgun sequencing applied to focal infection specimens. Accurate pathogen detection is essential to guide clinical management of focal infection, and studies have reported the use of mNGS in identification of causative agents in different kinds of focal tissues.<sup>21,22,24–26</sup> Therefore, the overall pro and cons of the usage of mNGS during clinical approach to focal infections is much worth evaluating.

In our study, we enrolled a variety of clinical specimens from human brains, lungs, hearts, livers, bones, skin and soft tissues, etc. Results revealed that positive percent agreement of mNGS compared to clinical diagnosis (86.30%) was significantly higher than that of conventional methods and culture (57.53%, 45.21%,  $P < 0.0125$ ), and mNGS also had a higher NPV than culture, highlighting that mNGS could be widely administered in the clinical approach to focal infections. In culture negative focal infections, mNGS can achieve an additional pathogenic diagnosis in 85.00% of the patients. While in culture positive samples, metagenomics can identify known pathogens in about 90.91% of focal infection, whose results were similar to the identical rate in the published articles.<sup>21,27</sup> One reason for mNGS's high diagnostic value may be that the mechanism of mNGS is to detect fragments of the causative agents while culture requires assistance of viable microorganisms. Another reason is that some patients had already been effectively treated in previous hospitals, which in our study led to a decreasing trend in culture positive percent agreement.

In USA, SSTI accounted for more than 14 million outpatient visits<sup>28</sup> and around 10% hospital admissions for infections.<sup>29</sup> Skin and soft tissues infections account for 42% of the enrolled patients, and the analysis showed that positive percent agreement of mNGS is statistically higher than that of culture (90.32% vs. 54.83%,  $p < 0.0125$ ). In a retrospective study in the USA,<sup>30</sup> culture had a 54.3% positive rate, similar to that reported in our study. These results all suggested that mNGS might hold great potential in both microbiological diagnoses of focal infections and the exclusion of non-focal infections.

Four false negative and three false positive cases were observed in our study. The miss detection of *Actinomyces europaeus* and *Fusobacterium necrophorum* were due to lack of specific sequence in our reference database, using multiple analytic tools is as important as upgrading of the data library to overcome deficiencies of individual methods or databases. The false positivity during mNGS could be due to factors including contaminant pathogen DNA across samples during mNGS library preparation, low-complexity sequences matching low-quality reads from the sample, misannotated species, or contaminants from database entries that also contain reads to human DNA, sequencing adaptors, or vectors, colonization. This highlighted that when using mNGS platform, false positive results might be specially alerted and repeated sequencing might sometimes be necessary.

The study had also discovered that standard unique reads of MTB complex, *Salmonella Typhimurium* and Brucellosis were relatively lower than other pathogens in our study. One explanation is that obtaining of intracellular bacteria's circulatory genome DNA might be difficult. *Nocardia*, although not an intracellular bacterium, belongs to the order of the Actinobacteria. The cytomembrane and cytoderm of the Actinobacteria is characterized by the combined features of fungus and bacteria and therefore express characteristics of relatively hard-to-break cell walls. Previous study has shown that specific RNA design can enrich the amplification of targeted pathogen genes and therefore assist mNGS to achieve higher unique reads.<sup>31</sup> This suggests that specific target enrichment system combining with mNGS might improve the detection rate of intracellular bacteria.

The study further compared turnaround time for mNGS, critical value report, strain culture and identification in Huashan Hospital. The workflow of the mNGS could be break into several part: the sample processing and nucleic acid extraction (7 h), library preparation (7 h), mNGS sequencing (14 h) and data analysis (3 h). The intermittent time would take less than 24 h. Overall, the turnaround time of each mNGS procedure ranges from 31 h to 55 h, which is shorter than the time most laboratory strain identification needs. However, mNGS performed in our study couldn't provide further drug susceptibility information.

MALDI-TOF can provide pathogen identifications in most isolates within one day,<sup>6</sup> but some slow-growing bacteria like *Actinomyces* may take up to 5 days.<sup>32</sup> The technique can only directly target a few types of clinical specimens (i.e. urine) and mainly relies on the successful culture of isolates. Hypothesis-driven multiplex PCR assays (i.e. GeneXpert, Filmarray),<sup>33</sup> can provide rapid identification of pathogens (less than one day), but they may miss less common or rare pathogens. Therefore, NGS may hold great future potential in the rapid diagnosis of the causative agents during infection.

We partitioned background sequences into two categories, one of which is those with no previous report in specific organ/tissue infections, and the other kind of pathogens exist widely in the environment and may colonize in certain body regions. To avoid missing novel pathogens, among the acquired background sequencing results, the clinicians would carefully examine patients' medical history, infection routes and infection sources to evaluate the possibility of novel pathogens.

Overall, mNGS may hold potential advantages in the following circumstances. First, it might offer critical assistance during the identification of causative agents that hadn't been known to infect human beings, such as Suid herpesvirus caused endophthalmitis.<sup>34</sup> Secondly, mNGS may assist diagnosis of pathogens that either had a relatively low culture rate or demanded a time-consuming culture process, such as intracellular pathogens, *Mycobacterium* family, etc. Successful isolation of intracellular pathogens has been based primarily on cell culture systems which had a relatively low culture rate, but now establishment of axenic culture media could partly improve culture rate and assist the study of intracellular pathogens' pathogenicity, virulence, and antibiotic susceptibility.<sup>35</sup> However, it sometimes is only limited to a certain type of intracellular pathogens, and mNGS may compensate for this shortcoming.

We recognize some limitations of this study. Failure to generate enough data in some samples, and limitation of the mNGS reference database can lead to failure of microorganisms detection using a metagenomics approach. Additionally, RNA was not sequenced in all of specimens, so some rare causes of focal infections such as viruses may be missing. Last, as our study was a single center retrospective cross-section, the enrollment of patients may have the potential to introduce some bias during clinical analysis.

## Conclusion

We conducted a focal infection cohort and evaluated the diagnostic ability of mNGS in focal infection. mNGS could be administered on a variety of focal tissues and provide credible results during suspected focal infections. The positive percent agreement of mNGS compared to clinical diagnosis is significantly higher than that of conventional methods and culture, resulting in a notably extra increase in pathogen identification. Antimicrobial treatment prior to sample collection may decrease culture positive percent agreement while that of mNGS seemed not to be affected. mNGS may hold extra diagnostic advantage in pathogens that are relatively slow-growing or hard to cultivate and time consumption of mNGS is shorter than conventional culture and strain identification. In future, additional clinical trials will be needed to evaluate the clinical usage of mNGS further and data analysis strategy could be also improved.

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## Declaration of Competing Interest

Authors had no potential conflicts of interest.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:[10.1016/j.jinf.2019.08.012](https://doi.org/10.1016/j.jinf.2019.08.012).

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