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Letter to the Editor

Identification of musculoskeletal infection with non-tuberculous mycobacterium using metagenomic sequencing

Dear Editor,

We read with interest the article by Guo et al.[1], in this Journal, "The advantages of next-generation sequencing technology in the detection of different sources of abscess". The paper stated that metagenomic next-generation sequencing (mNGS) could offer unbiased and rapid identification of causative pathogens from abscesses. A great diversity of pathogens can cause musculoskeletal infection and many of them cannot be cultured or identified by conventional methods. We hold the view that mNGS technology can also provide a sensitive diagnosis of musculoskeletal infection. Here, we discuss a series of musculoskeletal infection cases that illustrate the utility of using mNGS to detect non-tuberculous mycobacterium (NTM). The turn-around time of each mNGS test (including sample processing, sequencing and data analysis) was performed within 48 hours. Clinical data and mNGS results are summarized in Table 1.

Case 1, an 86-year-old male had undergone a right total hip arthroplasty eleven years prior. He received intraarticular injection in order to treat pain of right hip two years prior. After aggravate pain for two months, a sinus tract was presented at the incision. When admitted, his C-reactive protein (CRP) was 67.40 mg/L and erythrocyte sediment rate (ESR) was 96 mm/h. Synovial fluid white blood cell count was $11{,}929 \times 10^6$/L with 72.4% neutrophils. Acid-fast smear and aerobic/anaerobic bacterial and fungal cultures were all negative. Analysis of genomic DNA from synovial fluid using mNGS showed *Mycobacterium abscessus* with 2 reads. The patient underwent resection of his THA and aggressive debridement. *Mycobacterium abscessus* were identified from purulent synovial fluid and periprosthetic tissues obtained intraoperatively after four-days cultivation. Sonicated procedure was applied to the resected prosthesis.[2] The mNGS also detected *Mycobacterium abscessus* with 462 reads from sonicated fluid. The patient completed a six-week course of cefoxitin, amikacin and clarithromycin and his CRP and ESR declined gradually to normality. The incision was healing well.

Case 2, a 70-year-old female had undergone intraarticular injection in the right knee repeatedly due to osteoarthritis. One month later, she had gradually worsening pain on the knee, and low-grade fever. She was referred to us with a burst skin lesion on the knee (Fig.1A), and serum CRP of 44.4 mg/L and ESR of 121 mm/h. The X-ray and MRI showed massive joint effusion and destructed tibial plateau and femoral condyle (Fig. 1B,C). Synovial fluid analysis revealed $171{,}609 \times 10^6$/L with 60.1% neutrophils. However, acid-fast smear and cultures were all negative. The analysis of synovial fluid by mNGS suggested *Mycobacterium abscessus* (Fig. 1E). A week after intravenous administration of amikacin and cefclidine combined with oral clarithromycin, debridement operation was performed. Following a four-week course of the original therapeutic treatment after the operation, the anti-infection regimen was changed to oral clarithromycin alone. The patient was followed-up for six months and her CRP, ESR decreased gradually to normality. The incision was healing well (Fig. 1D).

Case 3, a 51-year-old male who had undergone the open reduction and internal fixation of the fracture of the left patella two months ago and the incision was purulent for more than one month. After his referral to our institution, his blood routine test showed CRP was 16.60 mg/L, ESR was 42 mm/h. The Acid-fast smear and cultures from multiple deep tissues were all negative. The result of mNGS from tissue revealed *Mycobacterium smegmatis*. After a four-week course of intravenous administration of levofloxacin and amikacin, his wound healed. The patient was followed up regularly and the inflammatory indicators gradually normalized.

Case 4, a 45-years-old male fish farmer had been pricked by a ray two months earlier. The skin of the second metacarpophalangeal joint of the left hand formed a deep ulcer, which connected to the metacarpophalangeal joint. The infection could not be controlled by debridement and empirical antibiotic therapy at other institutions. However, again, the acid-fast smear and cultures were all negative. *Mycobacterium ulcerans* with 8 reads was detected in the deep tissue by mNGS. The patient was treated with oral clarithromycin for 5 weeks, and the skin lesion healed.

The NTM can rarely cause musculoskeletal infection, which was commonly misdiagnosed as bacterial or tuberculosis infection for the absent of typical clinical manifestation.[3] Improper anti-infection and surgical treatment can lead to deteriorating cartilage and bone damage, non-healing incision and even amputation.[4] However, some species of NTM are difficult to grow on agar or chocolate plates. Meanwhile, NTM is easily establishes biofilms on implants, which makes it more difficult to be cultivated.[5]

mNGS is a novel, culture-independent technique that can provide rapid and unbiased identification of microorganisms with known genome from clinical sample.[1,6] mNGS has been proven to be useful in improving the detection rate of the pathogens that cause nervous system infections, sepsis, and periprosthetic joint infections.[7–9]

All the cases presented here were musculoskeletal infections that had the poor response to empirical antibiotic therapy and debridement. With mNGS detection, all four patients were treated with targeted antibiotics and obtained the satisfactory therapeu-

Abbreviation: mNGS, metagenomics next-generation sequencing; NTM, non-tuberculous mycobacterium; CRP, C-reactive protein; ESR, erythrocyte sediment rate; MRI, magnetic resonance imaging; PJI, periprosthetic joint infection.

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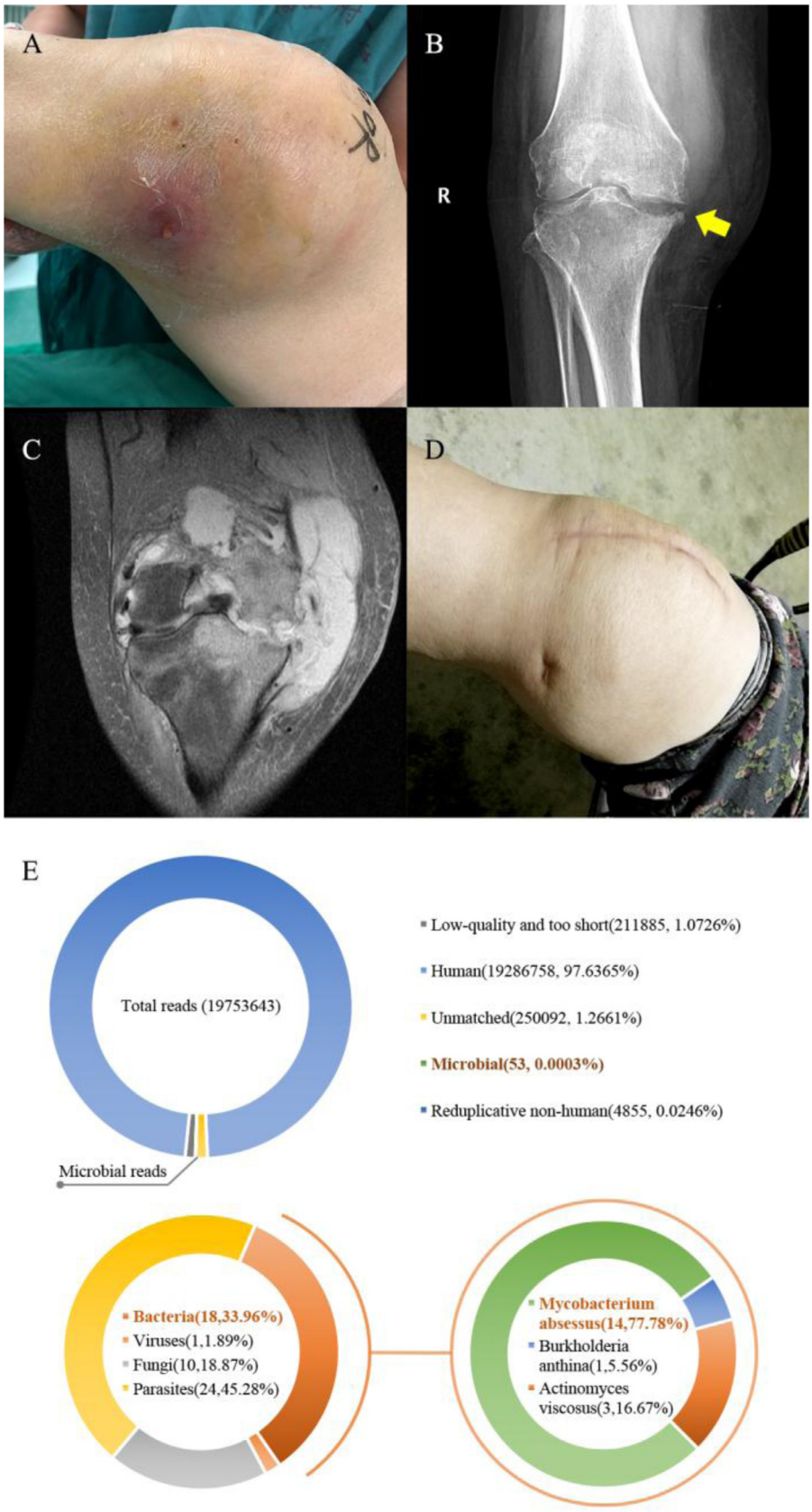

Fig. 1. (A) Swollen knee with burst skin before surgery. (B) X-ray film showed destructed medial tibial plateau and femoral condyle of right knee (yellow arrow). (C) A coronal T2-weighted magnetic resonance image of right knee showed massive joint effusion and destructed medial tibial plateau and femoral condyle. (D). The wound healed well without sinus and redness after debridement surgery. (E). The metagenomics next-generation sequencing result revealed *Mycobacterium abscessus* as potential pathogen. Number of mapped reads and percentage in the parentheses.
Clinical manifestation, radiological finding and metagenomics next-generation sequencing result of second case.

Table 1
Clinical data and mNGS results of four non-tuberculous mycobacteria musculoskeletal infection cases.

No.	Age	Site	Course	Culture results	mNGS results			Treatment
					Specimen	Bacteria	Reads	
1	86	Hip	1 year	Mycobacterium abscessus	Synovial fluid Sonication fluid	Mycobacterium abscessus Mycobacterium abscessus	2 462	Cefoxitin Amikacin Clarithromycin
2	70	Knee	1 month	Negative	Synovial fluid	Mycobacterium abscessus	14	Cefoxitin Amikacin Clarithromycin
3	51	Knee	1 month	Negative	Tissue	Mycobacterium smegmatis	4	Amikacin Levofloxacin
4	45	Hand	2 months	Negative	Tissue	Mycobacterium Ulcerans	8	Clarithromycin

tic outcome, which highlights the accuracy of mNGS as a diagnostic tool. mNGS detected *Mycobacterium abscessus* preoperatively in the first case, which was consistent with the intraoperative culture result. mNGS revealed the microbiological result earlier, which led to the use of appropriate antibiotics ahead of surgery.

The results of our study showed that mNGS is effective in detecting NTM in various types of musculoskeletal samples, including synovial fluid, tissue and sonicated fluid, which allows surgeons to have more options in collecting specimens. One caveat is that the assigned reads from synovial fluid and tissue are relatively low (2–14). This is likely due to the low extraction rate of nucleic acid from NTM or small number of planktonic bacteria. However, we found that the assigned reads in the sonicated fluid was more than 200-fold times higher compared to synovial fluid. Thus, sonication might improve detection rates.

In summary, our findings suggest that mNGS can be used to rapidly detect NTM in musculoskeletal infections. These cases draw attention to NTM infection as a possible cause of musculoskeletal infection, especially in patients with negative culture results or poor responses to empirical antibiotic therapy and debridement. We conclude that mNGS is a powerful tool for revealing pathogenic microorganisms.

Declaration

The authors declare that the investigations were carried out following the rules of the Declaration of Helsinki of 1975 (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/), revised in 2008.

Conflict of interest

The authors declare that they have no conflicts of interest.

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PCR cycle threshold to assess a diagnostic stewardship intervention for *C. difficile* testing

Dear Editor,

We read with interest the article by Kamboj et al., who demonstrated that low *Clostridium difficile* real-time polymerase chain reaction (PCR) cycle thresholds of detection (C_T) were predictive of toxin enzyme immunoassay positivity and disease severity in oncology patients who showed a positive *C. difficile* nucleic acid amplification test (NAAT) result.[1] These findings are consistent with those reported in other studies that found *C. difficile* PCR C_T (i.e., $\leq$ 26.0-28.0) may be similar to the results obtained from the cell cytotoxicity neutralization assay (CCNA) and superior to those obtained from toxin enzyme immunoassay in differentiating clinical *C. difficile* infection (CDI).[2–5]

We previously reported the use of a computerized clinical decision support (CCDS) tool that led to significantly reduced NAAT testing and National Healthcare Safety Network (NHSN) surveillance CDI events in our institution.[6] On the basis of the report by Kamobj et al., we sought to determine whether C_T data contributed to the identification of patients with lower probability of the disease.

Positive GeneXpert (Cepheid, Sunnyvale, CA) NAAT results were analyzed retrospectively between January 2014 and June 2018. C_T values obtained from tests that were ordered appropriately, according to the CCDS tool, were compared with those obtained from tests categorized as inappropriate. Inappropriate orders were defined as patients identified by the provider through the CCDS tool (post-CCDS) as lacking diarrhea or signs/symptoms of CDI or automatically flagged as a duplicate test (pre- or post-CCDS). A very high C_T value was defined as > 30.85, which is shown to have a 98.7% negative predictive value of a negative CCNA and toxin EIA, and thus, it likely reflects colonization with low organism burden.[4]

We found that C_T values were significantly higher in the inappropriate test group than in the appropriate test group (median: 26.7 versus 24.8 cycles, Table 1). The strongest predictor of an increased C_T value was a duplicate of a negative test. Fig. 1 demonstrates that C_T values were increased in the inappropriate test group, with a clustering of very high C_T results.

These results support the use of our current CCDS-based strategy. It is difficult to ascertain whether the result of 22.2% of very high C_T values (> 30.85) obtained from the appropriate test (compared to the result of 34.0% of very high C_T values obtained from the inappropriate test) is acceptable or not. We hypothesize that refinement of the CCDS may further reduce the proportion of tests with very high C_T values. In addition, it should be noted that 35% of C_T values categorized as inappropriate (excluding duplicates of positives) were < 26.0, thus suggesting that the patients were misclassified as being at a low pretest probability for the disease. We feel that this supports the use of CCDS tools for diagnostic guidance during test ordering, while allowing clinicians to bypass the tool and order tests on the basis of their clinical judgment.

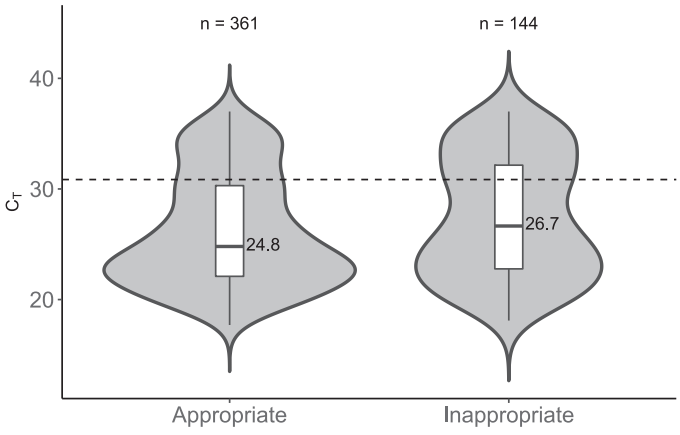

Fig. 1. Violin and box plots comparing C_T values between appropriate and inappropriate positive *C. difficile* NAATs. The dotted line depicts very high threshold = 30.85.

Although the absolute difference in the median C_T value between groups is relatively small, this likely reflects the prevalence of *C. difficile* colonization described among hospitalized patients (~4-29%) and the fact that colonized patients outnumber infected patients as 5 to 1.[7] Although we have not validated C_T values with CCNA at our institution, we found a similar association between C_T and toxin EIA described by Kamboj et al. and others, using a small (70 positive NAAT samples) set of historical internal validation samples (data not shown).

Considering the gold standard among *C. difficile* diagnostics, the CCNA assay is technically complex and labor intensive and has a slow turnaround time, thus making it impractical for routine clinical use. Unfortunately, *C. difficile* EIAs lack sensitivity. For these reasons, > 70% of hospitals currently use NAAT for the diagnosis of CDI.[8] The GeneXpert *C. difficile* PCR assay is highly sensitive at the manufacturer-set maximum C_T ($\leq$ 37.0), with an estimated detection limit of 1657 colony-forming units; however, a positive NAAT result alone may overdiagnose CDI up to half of the time.[2]

Analysis of C_T may offer a means to tailor *C. difficile* NAAT sensitivity and specificity according to various patient populations and levels of risk by modulating the C_T along a receiver operator characteristic curve. C_T also allows valuable feedback for diagnostic stewards, as we have shown. Validation of C_T for diagnostic and diagnostic stewardship purposes requires further research in various clinical settings before its clinical use can be widely applied.

Table 1
C_T values by order appropriateness.

	Total	Appropriate		Inappropriate		
		n (%)	Median C_T (IQR)	*n* (%)	Median C_T (IQR)	*P*
CCDS question						
Presence of diarrhea? (Appropriate Response = "Yes")	460	453 (98.5%)	24.9 (22.1–30.4)	7 (1.5%)	25.0 (22.2–29.9)	.847
Signs/Symptoms of CDI? (Appropriate Response = "Yes")	460	375 (81.5%)	24.9 (22.1–30.3)	85 (18.5%)	25.6 (22.3–31.2)	.393
Duplicate test*						
Duplicate of positive	1839	1799 (97.8%)	25.4 (22.4–30.7)	40 (2.2%)	27.3 (23.5–32.4)	.087
Duplicate of negative	1839	1825 (99.2%)	25.4 (22.4–30.7)	14 (0.8%)	31.6 (27.7–34.4)	.003
Inappropriate CCDS response or duplicate Test**	505	361 (71.5%)	24.8 (22.1–30.3)	144 (28.5%)	26.7 (22.8–32.2)	.023

P values were obtained by the Mann–Whitney U test. Duplicate of negative is defined as a negative result within 3 days of a previous negative result. Duplicate of positive is defined as a positive result within 14 days of a previous positive result.

* Three of the duplicate of negative and six of the duplicate of positive tests were performed post-CCDS implementation.

** Compared to all appropriate positive tests post-CCDS implementation.

Conflict of interests

None to declare.

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Hemagglutinin characteristics, changes in pathogenicity, and antigenic variation of highly pathogenic H7N9 avian influenza viruses in China

Dear Editor,

We read with interest a recent article about the evolutionary dynamics of H7N9 avian influenza viruses (AIVs) in mainland China in this journal.[1] Since 2013, five waves of the H7N9 epidemic have spread from eastern to southern and northern China. H7N9 was regarded as low pathogenic (LP) AIV in the previous four waves. In the 5th wave, some H7N9 viruses have an insertion of several basic amino acids at the HA cleavage site, which is associated with transition from low to high pathogenicity for chicken. In spite of vaccination practice, the pathogenicity and antigenicity of H7N9 high pathogenic (HP) AIVs in animal models remain unclear. In this study, we present a brief assessment of the characteristics, pathogenicity, and antigenicity of H7N9 HPAIVs in chickens and ducks.

During routine surveillance of AIV infection in poultry in 2017, we isolated 11 strains of the highly pathogenic virus, including 8 from chickens and 3 from ducks. The findings of sequence analysis of the HA gene from the isolates indicated four kinds of motifs at the HA cleavage site: PEVP**KG**↓**KR**TGLF, PEVP**KG**↓**KR**TA**R**↓GLF, PEVP**KRKR**TA**R**↓GLF, and PEVP**KRKR**NA**R**↓GLF. The motif PEVPKRKRNAR↓GLF was the first identified among the four and previously reported patterns.[2–5] The mutant strains exhibited a four-amino-acid (KRTA, KRNA or KRTK) insertion (339–342) and an I335V mutation at the cleavage site; some strains exhibited an additional G338R mutation (Table 1). It indicated that the motifs at the HA cleavage site of H7N9 HPAIVs are more varied than previously thought.

The receptor-binding sites (RBS) in the viral HA gene of HPAIVs possessed residues Q226 and G228 (H3 numbering), thus suggesting that these HPAIVs would preferentially bind to avian-like receptors.[6] All highly pathogenic isolates contained residues Q226 and G228 at the RBS in the viral HA gene (Table 1), except for the GDR1 virus, in accordance with the residues of most H7 LPAIVs. The GDR1 virus was isolated in January 2017, earlier than the remaining viruses in this study. The underlying mechanisms of virus evolution require further study.

Two isolates—a chicken (HBG1) and a duck (XZ2035) virus—were selected for pathogenicity studies in chickens and ducks.

We investigated the pathogenicity of HBG1 and XZ2035 in chickens by analyzing their IVPI. Both viruses were highly pathogenic in chickens, as demonstrated by the results of the IVPI assays, in which all inoculated chickens died within 2 days with IVPI values of 2.86 and 2.68, respectively (Supplement Table 1). After infection at a dose of 10^6 EID_{50}, both HBG1 and XZ2035 viruses were detected in the brain, heart, liver, spleen, lungs, intestine, and kidneys of inoculated chickens on days 2, 3, 4, and 5 post-infection (p.i.). Furthermore, virus shedding was detected from the tracheal and cloacal swabs of all dead chickens.

We also investigated the pathogenicity of HBG1 and XZ2035 in ducks by inoculation with 10^6 EID_{50} of each virus. Neither virus caused mortality or any symptom of infection within 14 days p.i., thus indicating that both viruses had low pathogenicity in ducks (Table 2). However, both viruses could be detected in the brain, heart, liver, spleen, lungs, intestine, and kidneys of inoculated chickens on days 3 and 5 p.i.. These results suggested that, although the HBG1 and XZ2035 viruses effectively replicated in the internal organs, they were not capable of causing death in ducks. Furthermore, all ducks inoculated with HBG1 and XZ2035 viruses exhibited virus shedding through their respiratory and digestive tracts and seroconversion (Table 2). Such shedding is an important source of infection to other poultry. In a previous study, no virus

Table 1
Molecular characteristics of the HA gene of H7N9 viruses isolated in this study.

Isolates	Virus strain abbreviation	Collection date	Amino acid sequence at cleavage site of HA	Receptor-binding sites in HA[a]	
				226	228
A/chicken/Guangdong/R1/2017	GDR1	7-Jan-2017	PEVPKGKRTAR↓GLF	L	G
A/chicken/Guangdong/1085/2017	G1085	22-Mar-2017	PEVPKGKRTKR↓GLF	Q	G
A/chicken/Guangdong/1245/2017	G1245	21-Mar-2017	PEVPKGKRTAR↓GLF	Q	G
A/chicken/Guangdong/1417/2017	G1417	21-Mar-2017	PEVPKGKRTAR↓GLF	Q	G
A/chicken/Tianjin/350/2017	TJ350	1-Jun-2017	PEVPKRKRTAR↓GLF	Q	G
A/chicken/Hubei/G1/2017	HBG1	1-Jun-2017	PEVPKRKRTAR↓GLF	Q	G
A/chicken/Anhui/1/2017	AH1	8-Jun-2017	PEVPKRKRNAR↓GLF	Q	G
A/chicken/Qinghai/2090/2017	QH2090	22-Nov-2017	PEVPKRKRTAR↓GLF	Q	G
A/duck/Jiangxi/1280/2017	J1280	25-May-2017	PEVPKRKRTAR↓GLF	Q	G
A/duck/Hubei/2129/2017	H2129	28-Nov-2017	PEVPKRKRTAR↓GLF	Q	G
A/duck/Xizang/2035/2017	XZ2035	2-Nov-2017	PEVPKRKRTAR↓GLF	Q	G

[a] H3 numbers were used throughout.

Table 2
Virulence and shedding of H7N9 highly pathogenic avian influenza viruses in ducks.

Isolate	Day post challenge	No. of necropsy	RRT-PCR for virus RNA							Virus shedding		Seroconversion (positive/total)
			Positive number/total number (Mean Ct)							Number shedding/total number (Mean Ct)		
			Brain	Heart	Liver	Spleen	Lung	Intestine	Kidney	Tracheal	Cloacal	
HBG1	3	5	5/5(30.5)	5/5(29.5)	5/5(29.2)	5/5(27.1)	5/5(28.2)	5/5(29.3)	5/5(27.3)	10/10(30.2)	10/10(28.7)	10/10
	5	5	5/5(30.1)	5/5(29.7)	5/5(29.5)	5/5(27.7)	5/5(27.8)	5/5(29.7)	5/5(27.1)	10/10(29.7)	10/10(28.3)	
XZ2035	3	5	5/5(30.3)	5/5(29.2)	5/5(29.7)	5/5(27.2)	5/5(28.5)	5/5(29.1)	5/5(26.8)	10/10(29.6)	10/10(28.4)	10/10
	5	5	5/5(29.7)	5/5(29.9)	5/5(29.3)	5/5(27.6)	5/5(28.0)	5/5(29.9)	5/5(27.5)	10/10(29.3)	10/10(28.1)	

shedding was detected in ducks inoculated with H7N9 LPAIVs.[7] The mechanisms of change in pathogenicity require further study.

Influenza viruses characteristically exhibit rapid antigenic drift. Therefore, we analyzed the antigenicity of the HBG1 and XZ2035 viruses relative to the H7 Re1 vaccine. The cross-reactive HI titers of H7 Re1 antiserum against the HBG1 and XZ2035 viruses were 3 log2 lower than that against the homologous H7 Re1 antigen (9 log2). In contrast, the cross-reactive HI titers of the antiserum against H7 Re1 antigens from the HBG1 and XZ2035 viruses were not obviously different from those against the two homologous H7N9 viruses (Supplement Table 2). These results indicate that the HBG1 and XZ2035 viruses exhibited antigenic drift to some extent, relative to the H7N9 vaccine strain.

Subsequently, we evaluated the protection efficacies of the inactivated reassortant H7 Re1 and rHBG1 vaccines. During the 10-day observation period, the H7-Re1- and rHBG1-vaccinated birds all survived and displayed no clinical signs of infection. In addition, no virus shedding was detected in tracheal or cloacal swabs from any of the experimental chickens on days 3 and 5 p.i. (Supplement Table 3). These results suggest that the current H7 Re1 vaccine provides complete protection against H7N9 HPAIVs. However, the antiserum against the HBG1 virus exhibited a broader spectrum of reactivity to other viruses. Therefore, the recombinant virus rHBG1 might be a better alternative for a vaccine.

In conclusion, the emergence of highly pathogenic H7N9 viruses in chickens is attributable to insertion and mutation of amino acids at the HA cleavage site, in which we identified multiform patterns. The circulation of highly pathogenic H7N9 viruses in poultry in China poses a new threat to the poultry industry, especially because of the antigenic drift of these viruses relative to the current vaccine. The measures undertaken by the government of China, including mass vaccination (for all kinds of poultry), surveillance, and culling, have played an important role in controlling H7N9 virus infection, as evident from the dramatic decrease in cases of infection among humans and poultry. More proactive and stringent measures should be undertaken in the future to prevent the spread of infection by the devastating highly pathogenic H7N9 viruses in poultry.

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Conflicts of interest

There are no potential conflicts of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2018.09.009.

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Next generation sequencing for diagnosis of severe pneumonia: China, 2010–2018

Dear Editor,

We read with interest the recent review by Brown et al.[1] that addressed the capability of next generation sequencing (NGS) in diagnosing suspected infectious encephalitis, and discuss the feasibility for introduction of NGS methods as a frontline diagnostic test. There is increasing evidence of a role for NGS in the work-up of undiagnosed encephalitis. Lower costs and increasing accessibility of these technologies will facilitate larger studies of these patients. They recommend NGS should be considered as a front-line diagnostic. Severe pneumonia, characterized by its complexity and lack of predictability, is one of the most common diseases in intensive care unit. It usually involves the dysfunction of other organs and thus carries high mortality. According to an epidemiologic report, the incidence of severe pneumonia has increased gradually in recent years. Despite the advance of clinical treatment, the mortality rate of severe pneumonia remains as high as 30–50%.[2] Since the NGS technique was newly introduced into clinical use, most research papers currently available are case reports with only a few conducting systematic evaluation. In this study, we will discuss the potential of NGS in evaluating severe pneumonia as well as its role in guiding precision therapy.

This retrospective observational study was conducted between January 2010 and June 2018.We reviewed the medical records of patients with severe pneumonia admitted at the intensive care unit (ICU) of Shanghai General Hospital. This study was approved by the institutional review board of the Shanghai general hospital. Since the data were anonymous, the need for informed consent was waived. The inclusion criteria were patients age between 18 and 85 years who presented with severe pneumonia; the severe pneumonia diagnosis was made according to the IDSA/ATS.[3] Exclusion criteria: noninfectious pneumonia; having severe immunosuppression, active tuberculosis, or end-stage diseases, or with a written “do not resuscitate” order; patients with a history of allergies to antibiotics or other drugs. A diagnosis of severe pneumonia was made by IDSA/ATS criteria[3] and the guidelines for the management of severe pneumonia issued by the IDSA/ATS guidelines. The primary outcome was the 28-day mortality, the second outcome were the 90-day mortality, the incidence of MODS, shock, AKI, length of hospital stay (days), duration of mechanical ventilation (days), CRRT, use of vasoactive agent**.** Control Group: Sputum, blood and BAL fluid samples were collected and sent for microbiologic testing before empiric antibiotics were administered. Antibiotic regimens were adjusted later based on the results of microbiology tests. The NGS group: Prior to administration of empiric antibiotics, sputum, blood and BAL fluid samples were collected and sent for conventional microbiologic testing; in the meantime, sputum, blood, and BAL fluid samples were also collected and sent to Beijing Genomics Institute for pathogen detection using NGS technique. Antibiotic regimens were adjusted later based on the results of NGS pathogen testing.

A total of 256 patients were screened for this study. 78 patients were excluded based on exclusion criteria at ICU admission. 178 (69.5%) patients were included. 48(27.0%) patients got NGS detection, and 130 (73.0%) patients got conventional detection. For patient demographics and baseline characteristics, there were no significant differences in sex, age, APACHE II scores, SOFA scores, COPD, Diabetes, Stroke, Bronchiectasis, Pulmonary fibrosis, Rheumatoid arthritis diagnosis.

Patient clinical characteristics were presented in Table 1. In comparison, the 28-day mortality of the NGS group was significantly lower than the control group (16.7% vs 37.7%, $p = 0.008$),the 90-day mortality of the NGS group was also significantly lower than the control group (16.7% vs 42.3%, $p = 0.002$).In Figs. 1 and 2, it also showed that 28-Days and 90-Days Survival proportions of NGS group were significantly higher than control group ($p = 0.0098$ and $p = 0.0033$ by log-rank test). There were no significant differences between the groups in complications (MODS, Shock and AKI), length of stay in hospital, duration of mechanical ventilation, and there were no significant differences in use of ECMO, CRRT, or vasoactive agents (Table 2). The most frequently isolated microorganisms in severe pneumonia patients were Acinetobacter baumannii (12.3%), Pseudomonas aeruginosa (10.1%), Staphylococcus aureus (5.6%), virus (17.4%), and fungus (11.8%) (Table 2). Compared with conventional microbiologic test, NGS showed a significantly

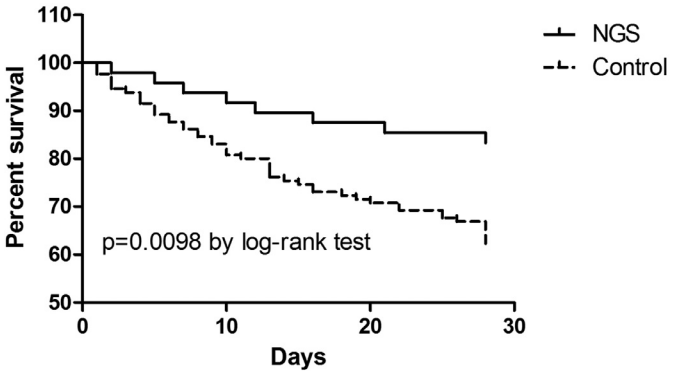

Fig. 1. 28-days survival proportions.

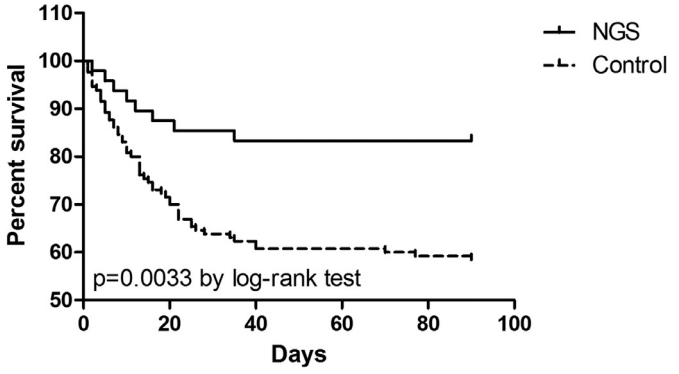

Fig. 2. 90-days survival proportions.

Table 1
Clinical data – NGS group vs control group.

	NGS $n=48$	Control $n=130$	P
Virus	11 (22.9%)	21 (16.2%)	0.297
Bacteria	33 (67.8%)	59 (45.4%)	0.006*
Fungi	3 (6.25%)	18 (13.8%)	0.199
28-day mortality	8 (16.67%)	49 (37.7%)	0.008*
90-day mortality	8 (16.67%)	55 (42.3%)	0.002*
HLOS(days)	18.07 ± 9.24	19.05 ± 17.38	0.770
Duration of mechanical ventilation (days)	8.48 ± 10.08	7.55 ± 5.91	0.636
AKI	9 (18.8%)	19 (1.46%)	0.501
MODS	28 (58.3%)	84 (64.6%)	0.441
Shock	13 (27.1%)	35 (26.9%)	0.983
CRRT	8 (16.7%)	15 (11.5%)	0.365
Use of vasoactive agent	13 (27.1%)	35 (26.9%)	0.983
ECMO	2 (4.17%)	7 (5.38%)	1

The detection of respiratory tract common virus is by direct immunofluorescence not by PCR. HLOS, hospital length of stay; AKI, acute kidney injury; MODS, multiple organ dysfunction syndrome; CRRT, continuous renal replacement therapy; ECMO, extracorporeal membrane oxygenation. $P > 0.05$, no statistical difference; $P < 0.05$, statistical difference.

Table 2
Pathogenic bacteria detected – NGS group vs control group.

	NGS $n=48$	Control $n=130$	P
Staphylococcus epidermidis	2	1	0.177
Streptococcus pneumoniae	8	0	<0.001*
Acinetobacter baumanii	2	20	0.069
Aspergillus	3	6	0.704
Pseudomonas aeruginosa	3	15	0.406
Legionnella	2	2	0.294
Staphylococcus aureus	3	7	0.731
Haemophilus influenzae	8	0	<0.001*
Klebsiella pneumoniae	3	3	0.346
Prevotella melaninogenicus	3	0	0.019*

* $P > 0.05$, no statistical difference; $P < 0.05$, statistical difference.

higher positive rate in detecting pathogenic bacteria (68.7% vs 45.4%, $p = 0.006$). NGS especially showed a significantly higher positive rate in detecting Streptococcus pneumoniae (16.7% vs 0%, $p < 0.001$), Haemophilus influenza (16.7% vs 0%, $p < 0.001$),and Prevotella melaninogenicus (6.3% vs 0%, $p = 0.019$).However, there was no difference between two techniques in fungal detection.

The main finding of this study is that the use of NGS was independently associated with reduced mortality of severe pneumonia: 16.67% vs 42.3%. Shorter HLOS were found in patients in NGS group compared with those who did not get NGS detection. Severe pneumonia is known for its severity and complexity. The correct use of targeting antibiotics relies on culture and drug sensitivity reports, which may take longer time according to the conventional detection methods.[4] Hence, empiric antibiotics are usually given as initial antimicrobial management. Empiric antibiotic treatment is usually lack of targeting antibiotics, the broad spectrum might lead to the emergence of antibiotic resistance. In other words, the targeted anti infection treatment should be given as soon as possible, once we acquire the cultural results. Prior study reports that the delay of the antibiotics use will increase the risk of death.[5] NGS can take up to 48 h (average of ~24 h) to generate the sequence data alone which is shorter than conventional detection methods (average of 3–5 days).[6] This is may the main reason that the use of NGS was independently associated with reduced mortality of severe pneumonia. Early targeted anti infection treatment is very important to reduce the risk of death.

In pathogenic bacteria detected positive rate, we found that NGS was also superior to conventional detection methods. In total, 79 samples (from 48 severe pneumonia patients in NGS group), including 10 sputum samples, 25 BAL fluid samples, and 48 blood samples, were sent for NGS testing. The results of NGS, compared with conventional microbiologic tests, are explained as follows:

The results of 25 NGS tests accorded with conventional microbiologic tests, with 13 samples positive and 11 samples negative by both methods; 14 samples tested negative by conventional methods were tested positive by NGS. Overall, the accordance rate between the two methods was higher than 50%, but NGS showed higher sensitivity. The accordance rate of two methods in testing BAL fluid samples was 55.5%. We also found that NGS had advantages on the detection of Streptococcus pneumoniae and Haemophilus lnfluenzae.

To our knowledge this is the largest amount of data study on the pathogen diagnosis of severe pneumonia by using NGS. Some recent articles describe the utility of the assay for detection of respiratory pathogens directly from BAL specimens from human stem cell transplant and lung transplant recipients.[7,8] NGS can assists clinical doctors to diagnose respiration diseases with unknown reasons quickly and precisely, However, there are also many challenges, such as lack of universal standards of results analysis and common guidelines for report interpretation. Differentiation of the background pathogens with the real pathogens is still a problem in need of clinical symptoms and other methods.[9]

This study suggests that NGS may lead to more rapid and accurate diagnosis with better clinical prognosis than conventional detection methods in severe pneumonia in ICU. It first shows that NGS can offer etiology evidence quickly for severe pneumonia patients, guide the treatment of clinics, and finally reduce the mortality. In other words, clinics call for new molecular methods like NGS for the severe pneumonia patients, to realize the aim of personalized and precision therapy while NGS can detect the pathogens quickly and is able to guide the antibiotics use.

Ethics approval and consent to participate

Ethics approval was obtained from Shanghai General Hospital Institutional Review Board (reference number [2018] KY004).

Consent for publication

All the authors agree to publish.

Conflict of interests

The authors declare that they have no competing interests.

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Authors' contributions

All the authors fulfill all three authorship criteria: conception and design or analysis and interpretation of data, drafting the article and revising it critically for important intellectual content and the final approval of the version to be published. All the authors read and approved the final manuscript.

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A cluster of cases of pneumocystis pneumonia identified by shotgun metagenomics approach

Dear Editor,

We read with interest the recent letter by Guo et al.[1] that addressed the capability of metagenomics sequencing in diagnosing different sources of abscess cases and agreed with that metagenomics sequencing could assist clinical decision with minimized turn-around time and satisfying diagnostic performance to detect multiple organisms.[2] Pneumocystis pneumonia (PCP) is a serious and sometimes critical infections caused by *Pneumocystis jirovecii*, which usually occurs in immune-suppressed patients accompanied with mixed infections, and early rapid diagnosis is critical in the disease prognosis. Here we reported 13 cases of PCP identified through shotgun metagenomics sequencing and compared its diagnostic value with conventional laboratory methods.

All 13 enrolled patients had pneumonia of unknown etiology. 3 patients had lymphoma and 4 patients had pemphigus. Case 2, a 68-year-old female, had several underlying diseases including breast cancer, liver cirrhosis and renal insufficiency. Other five patients each had renal transplantation, nephrotic syndrome, adult onset still's disease, prostate adenocarcinoma and aplastic anemia respectively. Notably, history of oral-glucocorticoids usage was observed in all patients and 6 patients were using other immunosuppressants while 3 patients were receiving chemotherapy during disease onset. All 13 patients received chest CT scans and had pulmonary infiltrates with or without pleural effusion. During hospitalization, Case 1 and 2 progressed to respiratory failure.

We conducted metagenomics sequencing in 15 samples collected from enrolled patients including three blood samples and 12 respiratory samples. The respiratory samples consisted of one tissue sample, seven bronchoalveolar lavage fluid (BALF) and four sputum samples. Both BALF and sputum samples were obtained from Case 10,12. The baseline characters obtained from patients were listed in Table 1.

Overall, metagenomics sequencing showed satisfying PCP detection rate compared to conventional methods. *Pneumocystis jirovecii* was detected in all samples, while for conventional methods, 4 out of 8 BALF samples had positive Wright–Giemsa stained findings and 1 out of 13 sputum sample had positive *Pneumocystis jirovecii* microscopy identification (Table 1). Notably, in case1-3, physicians diagnosed PCP through detection of *Pneumocystis jirovecii* sequencings reads in peripheral blood rather than respiratory samples, combined with patient's clinical manifestations and radiological findings.

Mixed-infections were observed in 11 patients. Metagenomics sequencing identified *Human herpes virus 5* in Case 1,2,3,7,9, *Human herpes virus 4* in Case 5,8,12 and *Herpes simplex virus*

Abbreviations: PCP, pneumocystis pneumonia; BALF, bronchoalveolar lavage fluid; CNS, central nervous system; NAAT, nucleic acid amplification testing; PCR, polymerase chain reaction; HE, hematoxylin-eosin.

Table 1
Baselines characteristics and metagenomics sequencing results of participants.

Case no.	Gender	Age	Sample type	Underlying diseases	Laboratory test(smear and culture)	β-D-glucan assays (pg/ml)	metagenomics sequencing results and specific reads(n)
1	Male	68	Blood	Lymphoma	Blood culture: *Staphylococcus aureus* Sputum Wright-Giemsa stained smear: fungi Sputum culture: *Candida*	<31.25	*Pneumocystis jirovecii* (427) *Staphylococcus aureus* (2) *Human herpes virus 5* (652) *Candida albicans* (2)
2	Female	68	Blood	Breast cancer; Liver cirrhosis; Diabetes; Renal insufficiency; Cardiac insufficiency	Blood smear and sputum Wright-Giemsa stained smear: negative; Blood and sputum culture: negative	<31.25	*Pneumocystis jirovecii* (5) *Candida tropicalis* (3791) *Human herpes virus 5* (38) *Aspergillus fumigatus* (3)
3	Male	62	Blood	Lymphoma	Blood smear and sputum HE stained smear: negative; Blood and sputum culture: negative	<31.25	*Pneumocystis jirovecii* (5) *Human herpes virus 5* (28)
4	Male	79	Lung tissue	Prostate adenocarcinoma	BALF Wright-Giemsa stained smear: *Pneumocystis jirovecii*; Sputum culture: *Candida albicans*	<31.25	*Pneumocystis jirovecii* (28) *Pseudomonas aeruginosa* (4179) *Candida albicans* (2)
5	Female	46	BALF	Adult onset still's disease	BALF Wright-Giemsa stained smear: *Pneumocystis jirovecii;* BALF and sputum culture: negative	206.28	*Pneumocystis jirovecii* (202) *Human herpes virus 4* (992)
6	Male	67	Sputum	Nephrotic syndrome	Sputum HE stained smear: negative; Sputum culture: negative	<31.25	*Pneumocystis jirovecii* (28)
7	Female	42	BALF	Pemphigus; Thymoma	BALF and sputum Wright-Giemsa stained smear: negative; BALF and sputum culture: negative	<31.25	*Pneumocystis jirovecii* (9) *Nocardia cyriacigeorgica* (42) *Human herpes virus 5* (187)
8	Male	56	BALF	Pemphigus; Severe pneumonia; Diabetes	BALF Wright-Giemsa stained smear: *Pneumocystis jirovecii;* BALF and sputum culture: negative	<31.25	*Pneumocystis jirovecii* (787) *Human herpes virus 4* (934) *Acinetobacter baumannii* (20,118) *Candida albicans* (14)
9	Male	39	BALF	Lymphoma	BALF and sputum HE stained smear: negative; BALF and sputum culture: negative	<31.25	*Pneumocystis jirovecii* (34) *Human herpes virus 5* (7)
10	Male	71	Sputum	Pemphigus	Sputum and BALF Wright-Giemsa stained smear: negative; BALF and sputum culture: negative	764.86	*Pneumocystis jirovecii* (773) *Human simple virus 1* (869)
			BALF				*Pneumocystis jirovecii* (621) *Human simple virus 1*(258)
11	Male	28	BALF	Renal transplantation	BALF Wright-Giemsa stained smear: *Pneumocystis jirovecii*; BALF culture: negative	<31.25	*Pneumocystis jirovecii* (9092)
12	Male	64	Sputum	Pemphigus	Sputum and BALF Wright-Giemsa stained smear: negative; BALF and sputum culture: negative	250.89	*Pneumocystis jirovecii* (253) *Human herpes virus4* (520)
			BALF				*Pneumocystis jirovecii* (644) *Human herpes virus4* (93)
13	Male	62	Sputum	Aplastic anemia	Sputum Wright-Giemsa stained smear: *Pneumocystis jirovecii;* Sputum culture: negative	82.1	*Pneumocystis jirovecii* (7) *Human simple virus 1* (2807) *Enterococcus faecium* (669)

1 in Case10,13. Furthermore, pathogens including *Enterococcus faecium, Nocardia cyriacigeorgica, Candida tropicalis, Aspergillus fumigatus* and etc. were additionally detected by high-throughput sequencing (Table 1).

We further explored the applicable cut-off values for metagenomics sequencing. Statistical analysis revealed that proportions of *Pneumocystis jirovecii* specific reads in fungi surpassed 85.00% in 13 out of 15 samples, and only two blood samples reported *Pneumocystis jirovecii* specific reads proportions below 85% (Fig. 1A). For Case 2 whose symptoms were in consistency with PCP, the ratio of *Pneumocystis jirovecii* reads was relatively low as patient also suffered from blood stream *Candida tropicalis* infections which accounted for the majority of fungi reads. 94.59% (35/37) of the detected pathogenic microbes ranked among top 15 in specific reads ranking (Fig. 1B), including *Pneumocystis jirovecii* reads counts in 14 samples. Although *Pneumocystis jirovecii* of Case 13 ranked 25 among all microbes, its proportion in fungi reached 100.00% and PCP was confirmed by sputum smear as well. Therefore, our study suggested that *Pneumocystis jirovecii* 's specific reads ranking among top15 or its relative reads proportion in fungi higher than 85% might be satisfactory cut-off values for clinical diagnosis of the disease.

PCP is a severe infection which poses threat to patients, especially those with immunity deficiency. Up till now, the definitive diagnosis of PCP relies on finding out *Pneumocystis jirovecii* in respiratory secretion, tissue or BALF sample. Wright–Giemsa stained smear is the most commonly applied method in past decades and other tinctorial methods including methenamine silver, cresyl

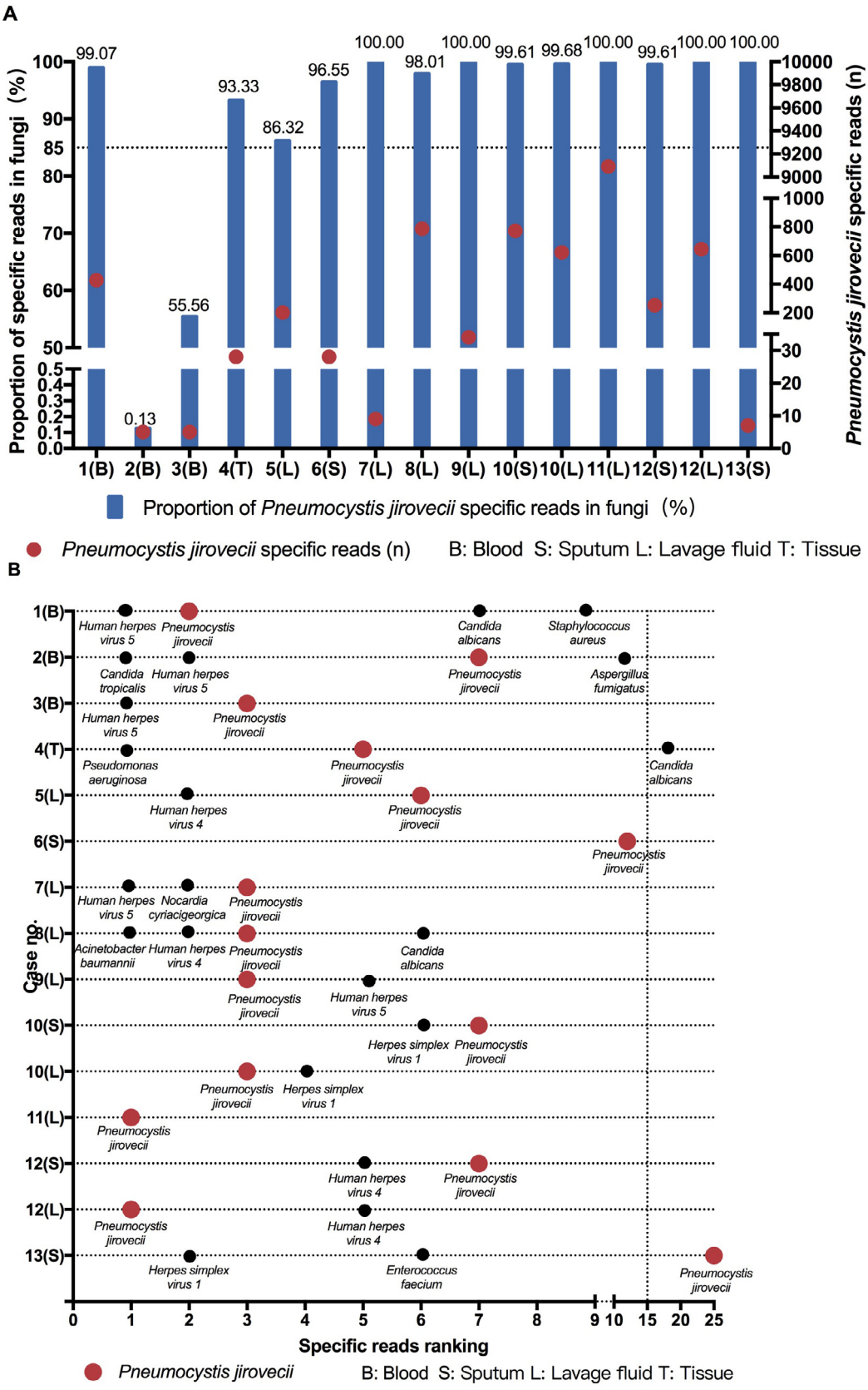

Fig. 1. A. *Pneumocystis jirovecii* specific reads and its proportions in fungi.
B. Specific reads ranking of detected pathogenic microbes in enrolled patients.

echt violet and fluorescence staining may further increase the detection sensitivity. In recent years, nucleic acid amplification testing (NAAT) and serologic biomarkers such as β-D-glucan and S-adenosyl methionine have also shown to benefit patients.[3,4]

Shotgun metagenomics sequencing offers a novel and unbiased method in the clinical approach to infectious diseases and our study revealed that high-throughput sequencing had a higher detection rate of PCP compared to conventional methods.

Despite the highly sensitive nature of metagenomics sequencing, one reason for the relatively lower positive rate in laboratory approach in our study may be that Wright-Giemsa stained smear were only performed in 10 respiratory specimens, and 5 patients didn't provide BALF either due to patient's refusal or unstable medical conditions for invasive procedures. Although studies have reported that fluorescence staining with monoclonal antibodies could increase the PCP diagnostic rate to 95% (5), the order of any specific tinctorial methods require prior clinical suspicions of certain pathogens and metagenomics sequencing might held the advantage as an all-in-one diagnostic tool in the clinical approach to pneumonia.

What's more, 3 patients who either couldn't withstand bronchoscope examination or declined invasive operation were diagnosed PCP through metagenomics sequencing of blood samples. One explanation might be that pieces of *Pneumocystis jirovecii* could penetrate through local infectious site and circulate into peripheral blood, especially when the patient's immune system is compromised. This suggests that in future, high throughput sequencing of the peripheral blood samples might be an alternative choice for patients with localized infectious lesions but couldn't tolerate invasive procedures.

Another advantage of metagenomics sequencing this study has revealed is its ability to detect co-infections including bacteria, virus, fungi and parasites in one single approach. Therefore, metagenomics sequencing might be more likely to benefit immune suppressed patients who are more likely to be infected by multiple pathogens.

In conclusion, our study originally illustrated application of shotgun metagenomics sequencing in diagnosing PCP and found that it had a satisfactory diagnostic value compared to conventional laboratory methods. Patients with mixed infections or those who could not withstand invasive procedures may be further benefited from this technique. The combination of specific *Pneumocystis jirovecii* reads ranking and its proportion rate might be a promising cut-off value in metagenomics sequencing. However, with the limit of sample size, further investigations and evaluation is necessary.

Declaration

We confirm that each individual named as an author meets the journal's criteria for authorship and neither the entire paper nor any part of its content has been published or accepted elsewhere. It is not being submitted to any other journal.

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A Conflict of Interest

All authors report no potential conflict of interest.

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Supplementary materials

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