Rapid Identification of Pathogens Associated With Ventilator-Associated Pneumonia By Nanopore Sequencing

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Abstract

BACKGROUND: Etiology detection is crucial in diagnose and treatment of ventilator associated pneumonia (VAP). However, the detection method is in need of improvement. In this study, we used Nanopore sequence to build a quick detection protocol and compared the efficiency of different methods on 7 common VAP pathogens.

METHODS: Endotracheal aspirate (ETA) of 83 patients with suspected VAP from Peking University Third Hospital were collected, saponin were used to deplete host genomes, and PCR or non-PCR library construction method were used and compared for sequencing. MinION and local data analysis methods were used for sequencing and data analysis.

RESULTS: Saponin depletion effectively removed 11 of 12 human genomes, while most pathogenic bacteria genome results showed no statistical difference except for *S.pneumoniae* who had a 0.31 times depletion. Meanwhile, the average sequence time reduced from 19.2hrs to 1.73hrs. Compared with PCR library construction method, the non-PCR method has a better average sensitivity (84.17% VS 81.35%) and specificity (97.42% VS 88.52%), while the non-PCR costs less time. The whole method takes 5-6hrs from ETA extraction to pathogen classification. After analyzed the 7 pathogens enrolled, the average sensitivity of metagenomic sequencing were about 2.4 times higher than that of clinical culture (91.79%VS 37.92%), and the average specificity was 98.08%.

CONCLUSIONS: Using saponin to remove human genome and non-PCR method to build library can be used for identification of pathogens in ETA of VAP patients within 6 hours by MinION, which provides a new approach for rapid identification of pathogens in clinical departments.

Background

Ventilator-associated pneumonia (VAP) refers to pneumonia that occurs between 48hrs after mechanical ventilation (MV) to 48hrs after extubating [1]. VAP is a common and serious complication of MV patients, leading to an increased mortality [1]. Studies have shown that timely and effective antibiotic treatment can significantly improve the cure rate of patients with VAP and reduce the risk of disease deterioration and death [2–4], which depends on rapid identification of pathogens. Timely pathogenic detection plays a crucial role in the process of disease diagnosis and treatment [2–4]. In China, the most common pathogenic bacteria of VAP include *Pseudomonas aeruginosa* (*Paeruginosa*), *Acinetobacter baumannii* (*A.baumannii*), *Klebsiella pneumoniae* (*K.pneumoniae*) and *Staphylococcus aureus* (*S.aureus*) [5, 6].

At present, the most commonly used pathogen detection method in clinical practice is bacterial culture [7]. However, bacterial culture requires 24-48hrs but it is not conducive to rapid and accurate identification of pathogens. Genomic identification of endotracheal aspirate (ETA) has become a new method for rapid identification of pathogens. qRT-PCR and PCR-based FilmArray (R) Panel method can quickly identify pathogens, but this method can only be used for specific pathogens and is not useful for detection of unknown pathogens [8–12]. Second-generation sequencing technology has the advantages of high throughput and sequencing analysis for unknown pathogens, but it also has high requirements for experimental equipment and cost, so the sequencing is difficult to be carried out in clinical laboratories [13]. Therefore, it usually takes 24hrs or more for second-generation sequencing from sample extraction to result acquisition.

The Nanopore sequencing can quickly identify DNA or RNA sequences in real-time. The MinION based on Nanopore sequencing technology can be used for DNA sequence detection only by connecting to a laptop, and the
detection results can be read and analyzed in real time, providing the possibility for clinical departments to carry out pathogen genome detection [14, 15]. Although it has been used in several labs to test samples of the lower respiratory tract, its methodology is not unified, the influence of different processing methods is also not clear [12, 16, 17].

Therefore, this study compared the detection efficiency of different methods for different pathogens and constructed a data analysis method suitable for clinical departments in Chinese hospitals based on local servers, which provided guidance and suggestions for the selection of pathogen identification methods for VAP patients.

**Methods**

**Patients**

105 patients over 18 years old who were admitted to the respiratory intensive care unit, critical care unit and emergency department of Peking University Third Hospital (PUTH) from September 2019 to December 2020 who were experiencing MV for more than 48hrs and suspected of having VAP were collected, 83 patients were finally admitted in this study (Flowchart 1). This study was proved by Ethics Committee of Peking University Health Sciences (IRB00001052) and the Ethics Committee of Peking University Third Hospital (M20200352).

**Sample collection**

Two ETAs from patients suspected to have VAP were collected within 24hrs, one performed microbiology culture in clinical laboratory of PUTH, one was taken to our lab for further research. 4X volume of PBS were added into ETA, pipette and separated into 1ml/tube. After centrifugation for 15mins in 8000rpm, the sediment was collected and snap frozen by liquid nitrogen, then stored in -80℃.

**Host depletion with saponin**

Sediments were resuspended with 250µl PBS, add 200µl 5% saponin (S0019, Tokyo Chemical Industry, Tokyo, Japan) and pipette. Samples were placed in room temperature for 10mins before add 350µl Nuclear-Free-Water (NF-Water) and place for another 30s, then add 12µl 5M NaCl and invert. Centrifugation in 4℃ 8000rpm for 5mins, discard supernatant and resuspend sediment with 100µl PBS, add 100µl HL-SAN Buffer (100mM MgCl₂ in 5M NaCl) and 10µl HL-SAN DNase (25,000 units, 70910-202, Articzymes, Tromso, Norway), shake in 37℃ for 15mins. Centrifugation in 4℃ 8000rpm for 5mins, discard supernatant and wash sediment with 1000µl PBS for twice. The same procedure was used in the control group, but all reagents were replaced by NF-Water (Flowchart 2).

**DNA extraction**

BSCC45S1E kits and GenePure Pro (Bioer Technology, Hangzhou, Zhejiang, China) were used for DNA extraction. Lysozyme was dissolved in TET Buffer and mixed by shaking. Add 180µL mixture to each sample and incubate for 30mins at 37℃ after shaking. Add 20µl ProteinaseK and sample to Columns 1 and 7 of the kit and place into the machine. DNA concentration and purity were determined by NanoDrop after extraction.

**Library build, sequencing and data analysis**

The undepleted DNA and non-PCR library build of depleted DNA was performed using rapid sequencing kit (SQK-RAD004, ONT, Oxford, UK) and rapid barcode kit (SQK-RBK004, ONT, Oxford, UK), while the PCR library build of depleted DNA was performed using rapid PCR barcode kit (SQK-RPB004, ONT, Oxford, UK). Non-PCR method was
performed following the instruction, 400ng DNA of each sample was used (when the maximum amount of 7.5µl DNA is less than 400ng, then add 7.5µl) for sequencing. The PCR method was carried out according to the instruction. 5ng DNA was used for each sample, the extension time was shortened from 6mins to 4mins, and the amplification cycle was increased from 14 cycle to 25 cycle [17]. Sequencing was performed using MinION (ONT, Oxford, UK) and R9.4 flowcells (FLO-MIN106D, ONT, Oxford, UK). Raw data collection and base-calling were performed using MinKNOW (v.19.12.5, ONT, Oxford, UK) and Guppy (v.3.2.10, ONT, Oxford, UK) software. The data were collected and analyzed in real time. Sequencing was continued for 1-2hrs after the pathogens causing VAP were identified. If there were no more pathogenic bacteria detected, the sequencing would be stopped.

The raw data generated by sequencing were filtered using NanoFilt (v.2.7.1) for joint sequence resection (– headcrop 150 –tailcrop 50) and low-quality segments (-q 7 -l 500), and NanoPlot (v.1.32.1) for filtration quality statistics and visualization. Minimap2 (v.2.17) was used to align the filtered clean FASTQ file with Human GRCh38 Genome (NCBI), SamTools (v.1.11) was used to extract the unaligned sequence (-f 4) and convert the generated data to FASTQ format. The Kraken2 (v.2.1.1) standard reference was used for sequence classification (Flowchart 3).

qRT-PCR

For the samples whose clinical culture results were inconsistent with the sequencing results, qRT-PCR was performed to identify the presence of the pathogen. For the depleted/undepleted samples, qRT-PCR was performed to detect human or bacterial genes. In each sample, 10µl SYBR Master Mix (11184ES08, Yasen, Shanghai, China), 7.2µl NF-water, 0.4µl Forward and Reverse Primer (synthesized by Beijing Ruibio Biotech Co., Ltd) (Additional File 1), and 2µl DNA samples were added. The disposable sputum collector was flushed with sterile normal saline, and DNA was extracted as negative control group. Bacteria from ATCC were extracted as positive control group of pathogens (Supplementary Table 1). DNA of A549 cell line was extracted and used as human genomic positive control group. PCR cycle was set as pre-incubation at 95°C for 2mins, amplification for 40 cycles at 95°C for 10s, 60°C for 30s, final melt curve was 95°C15s,60°C 60s,95°C15s. Results were analyzed using CT values.

Treatment of quality control strains

Standard strains of *S.aureus, A.baumannii, Stenotrophomonas maltophilia (S.maltophilia), Paeruginosa, Streptococcus pneumoniae (S.pneumoniae), Escherichia coli (E.coli) and K.pneumoniae* were obtained from ATCC (Additional File 1), monoclonal colonies were selected after cultured overnight and dissolved in normal saline to make suspension of 4.5 McF, then divided into 1ml/tube, centrifuged at 13000rpm at 4°C for 1min, collected precipitation, snap frozen by liquid nitrogen and stored at -80°C.

Statistical analysis

The qRT-PCR results were analyzed using T test, and the sensitivity and specificity used Binomial distribution. P value less than 0.05 was considered to have a statistical difference. R (v.4.0.3) and SPSS (v.19) was used for statistical analysis, and the tool [http://vassarstats.net/](http://vassarstats.net/) was used for sensitivity and specificity calculation. The image was produced using OriginPro 2017C (b8.4.2.380).

Results

Effect of saponin depletion and its influence on sequencing results
The DNA of S01, S02 and S03 patients' ETA were extracted for single sample library build and sequence. A new flowcell can only support 19.2 ± 3hrs sequencing, but the results were still dominated by human genome reads, and no clear pathogen causing VAP were found.

Therefore, we tried to remove the human genome using the depletion operation. 12 clinical samples were taken and divided into 2 parts equally. One part was treated with saponin depletion protocol, and the other with the same procedure but all reagents were replaced by NF-Water (Flowchart 2). According to Table 1 and Fig. 1, all the samples successfully removed part of the human genome, and 11 of them showed significant differences in the content of human genes between the depleted group and the undepleted group. Single sample sequencing was performed on the samples of the above 3 patients after saponin depletion. After sequenced for 1.73 ± 0.76hrs, pathogen detection results consisted with the clinical culture results were obtained (Additional File 2), suggesting that the sequencing efficiency can be greatly improved by depletion.
| Sample ID | Treatment | Human DNA assay (Ct mean ± SD) | Human depletion [\(\Delta C_t\) \(\text{mean}_{\text{Un} \text{depleted}}-\text{mean}_{\text{Depleted}}\)] | Human depletion (P value) | Bacteria DNA assay (Ct mean ± SD) | Bacteria concentration change [\(\Delta C_t\) \(\text{mean}_{\text{Un} \text{depleted}}-\text{mean}_{\text{Depleted}}\)] | Bacteria concentration change (P value) |
|-----------|-----------|-------------------------------|-----------------------------------------------|--------------------------|-----------------------------------|-----------------------------------------------|-------------------------------------------|
| S01       | Depleted  | 18.568 ± 0.160                | -0.51                                         | 0.004                    | 21.509 ± 0.055                    | -1.32                                         | < 0.001                                   |
|           | Undepleted| 18.059 ± 0.084                |                                               |                          | 20.186 ± 0.060                    |                                               |                                           |
| S02       | Depleted  | 19.629 ± 0.049                | -0.82                                         | 0.005                    | 20.693 ± 0.021                    | -1.91                                         | 0.001                                     |
|           | Undepleted| 18.876 ± 0.138                |                                               |                          | 18.784 ± 0.128                    |                                               |                                           |
| S03       | Depleted  | 28.750 ± 0.202                | -9.9                                          | < 0.001                  | 25.248 ± 0.211                    | -2.05                                         | < 0.001                                   |
|           | Undepleted| 18.843 ± 0.050                |                                               |                          | 23.190 ± 0.011                    |                                               |                                           |
| S04       | Depleted  | 30.507 ± 0.511                | -11.43                                        | < 0.001                  | 19.405 ± 0.169                    | -0.08                                         | 0.486                                     |
|           | Undepleted| 19.077 ± 0.067                |                                               |                          | 19.323 ± 0.015                    |                                               |                                           |
| S05       | Depleted  | 24.546 ± 0.084                | -6.35                                         | < 0.001                  | 19.693 ± 0.169                    | -1.13                                         | 0.017                                     |
|           | Undepleted| 18.197 ± 0.386                |                                               |                          | 20.827 ± 0.308                    |                                               |                                           |
| S06       | Depleted  | 28.146 ± 1.059                | -9.65                                         | 0.002                    | 26.523 ± 0.144                    | -5.93                                         | < 0.001                                   |
|           | Undepleted| 18.495 ± 0.403                |                                               |                          | 20.589 ± 0.204                    |                                               |                                           |
| S08       | Depleted  | 19.629 ± 0.086                | -0.75                                         | 0.001                    | 21.538 ± 0.053                    | 4.11                                          | < 0.001                                   |
|           | Undepleted| 18.876 ± 0.179                |                                               |                          | 25.652 ± 0.222                    |                                               |                                           |
| S10       | Depleted  | 25.587 ± 0.093                | -6.68                                         | < 0.001                  | 20.115 ± 0.286                    | -0.504                                         | 0.072                                     |
|           | Undepleted| 18.903 ± 0.421                |                                               |                          | 19.610 ± 0.164                    |                                               |                                           |
| S12       | Depleted  | 27.051 ± 0.199                | -8.21                                         | < 0.001                  | 24.641 ± 0.077                    | 4.48                                          | < 0.001                                   |
|           | Undepleted| 18.835 ± 0.129                |                                               |                          | 29.123 ± 0.185                    |                                               |                                           |
### Table 1

| Sample ID | Treatment | Human DNA assay (Ct mean ± SD) | Human depletion [Ct mean_{Depleted} - Ct mean_{Undepleted}] | Human depletion (P value) | Bacteria DNA assay (Ct mean ± SD) | Bacteria concentration change (Ct mean_{Depleted} - Ct mean_{Undepleted}) | Bacteria concentration change (P value) |
|-----------|-----------|-------------------------------|------------------------------------------------------------|---------------------------|-----------------------------------|--------------------------------------------------------------------|----------------------------------------|
| S13       | Depleted  | 31.984 ± 1.372                | -13.48                                                     | 0.003                     | 29.092 ± 0.055                    | -6.40                                                              | < 0.001                                |
|           | Undepleted| 18.505 ± 0.143                |                                                            |                           | 22.696 ± 0.150                   |                                                                    |                                        |
| S14       | Depleted  | 23.710 ± 0.319                | -3.84                                                      | < 0.001                   | 13.605 ± 0.084                    | 3.53                                                               | < 0.001                                |
|           | Undeplet| 19.869 ± 0.131                |                                                            |                           | 17.143 ± 0.067                   |                                                                    |                                        |
| S15       | Depleted  | 20.807 ± 0.838                | -1.34                                                      | 0.097                     | 14.208 ± 0.175                    | 2.50                                                               | < 0.001                                |
|           | Undepleted| 19.466 ± 0.282                |                                                            |                           | 16.705 ± 0.193                   |                                                                    |                                        |

However, whether saponin can also deplete bacteria is not clear. After the saponin depletion procedure, the bacterial DNA content of 5 samples were significantly increased (S05, S08, S12, S14, S15), 5 were significantly decreased (S01, S02, S03, S06, S13), 2 showed no significant difference (S04, S10) (Table 1, Fig. 1). In order to explore if saponin depletion progress could also deplete bacteria genomes, 7 cultured pathogens obtained from ATCC were used and divided into two parts equally, following the comparing procedure in Flowchart 2. As can be seen from Table 2, saponin depletion had no significant effect on pathogens except *S.pneumoniae*. The strain concentration of *S.pneumoniae* decreased by about 0.31 times after depletion.

### Table 2

| Pathogen         | Depleted (CT mean ± SD) | Undepleted (CT mean ± SD) | P value |
|------------------|-------------------------|---------------------------|---------|
| *P. aeruginosa*  | 13.172 ± 0.035          | 13.136 ± 0.065            | 0.455   |
| *S. aureus*      | 18.712 ± 0.070          | 18.517 ± 0.107            | 0.067   |
| *S. pneumoniae*  | 25.392 ± 0.095          | 17.529 ± 0.321            | < 0.001 |
| *K. pneumoniae*  | 18.995 ± 0.509          | 18.794 ± 0.227            | 0.580   |
| *S. maltophilia* | 11.955 ± 0.163          | 12.003 ± 0.204            | 0.767   |
| *A. baumannii*   | 14.388 ± 0.179          | 14.021 ± 0.143            | 0.053   |
| *E. coli*        | 12.357 ± 0.121          | 12.104 ± 0.081            | 0.096   |

### Influence of PCR and non-PCR methods on sequencing results

ONT provides two library building methods: the PCR mixed method and the non-PCR mixed method. Our laboratory compared the performance of two sequencing methods in terms of sequencing duration and sequencing results. As described in Table 3, in the 29 sequencing results, the non-PCR method performs better in non-PCR group than...
in PCR group (average sensitivity: 84.17% VS 81.35%, average specificity: 97.42% VS 88.52%) (detailed data in Additional File 3). In addition, the average sequencing time per sample for both methods were similar, but the PCR method required an additional 2hrs 16mins of amplification, so the non-PCR method takes less time overall. Based on the results of sequencing duration and performance, non-PCR method can be used as the first choice for sequencing.

### Table 3
Sensitivity and Specificity of non-PCR and PCR

| Pathogen        | Sensitivity (95%CI)      | Specificity (95%CI)     |
|-----------------|-------------------------|-------------------------|
|                 | Non-PCR | PCR            | Non-PCR | PCR            |
| *P. aeruginosa* | 80% (29.88% - 98.95%)   | 60% (17.04% - 92.74%)  | 100% (82.83% - 100%) | 95.83% (76.88% - 99.78%) |
| *S. aureus*     | 83.33% (36.48% - 99.12%) | 83.33% (36.48% - 99.12%) | 95.65% (76.03% - 99.77%) | 91.3% (70.49% - 98.48%) |
| *S. pneumoniae* | 80% (29.88% - 98.95%)   | 80% (29.88% - 98.94%)  | 95.83% (76.88% - 99.78%) | 91.67% (71.53% - 98.54%) |
| *K. pneumoniae* | 75% (35.58% - 95.54%)   | 85.71% (42% - 99.25%)  | 90.48% (68.17% - 98.33%) | 81.82% (59% - 94%) |
| *S. maltophilia*| 100% (31%-100%)         | 100% (31% - 100%)      | 100% (83.98% - 100%) | 96.15% (78.42% - 99.80%) |
| *A. baumannii*  | 87.5% (60.41% - 97.8%)  | 93.75% (67.71% - 99.67%) | 100% (71.66% - 100%) | 84.62% (53.66% - 97.29%) |
| *E. coli*       | 83.33% (36.48% - 99.12%) | 66.67% (24.11% - 94%)  | 100% (82.19% - 100%) | 78.26% (55.79% - 91.71%) |

### Data analysis

DNA extraction and sequencing identification of 83 samples were conducted according to the Flowchart 4, the whole procedure takes 5–6hrs from ETA extraction to obtain classification result. The identification results are shown in Additional File 2. As long as one of clinical culture or qRT-PCR was positive, the pathogen was recognized as existed. As indicated in Table 4, besides *Paeruginosa* and *K.pneumoniae*, the metagenomic sequencing sensitivity of other 5 pathogens were over 90%, which is far more than that of clinical detection. Although sensitivity of *Paeruginosa* and *K.pneumoniae* are under 90%, the metagenomic sequencing method is still more sensitive than clinical culture. The specificity of pathogens except for *K.pneumoniae* were over 98%.
| Pathogen        | Sensitivity (95%CI) | Metagenomic sequencing | Specificity (95%CI) | Metagenomic sequencing |
|-----------------|---------------------|------------------------|---------------------|------------------------|
|                 | Microbiology culture|                        | Microbiology culture|                        |
| P. aeruginosa   | 73.33% (44.83% − 91.09%) | 78.57% (48.82% − 94.29%) | 100% (93.34% − 100%) | 98.55% (91.11% − 99.92%) |
| S. aureus       | 20% (5.31% − 48.63%)  | 93.33% (66.03% − 99.65%) | 100% (93.34% − 100%) | 100% (93.34% − 100%)   |
| S. pneumoniae   | 0 (0% − 48.32%)      | 100% (51.68% − 100%)    | 100% (94.08% − 100%) | 98.7% (91.99% − 99.93%) |
| K. pneumoniae   | 60% (32.89% − 82.54%) | 86.67% (58.39% − 97.66%) | 100% (93.34% − 100%) | 92.65% (82.98% − 97.26%) |
| S. maltophilia  | 33.33% (12.99% − 61.31%) | 93.33% (66.03% − 99.65%) | 100% (93.34% − 100%) | 100% (93.34% − 100%)   |
| A. baumannii    | 78.79% (60.6% − 90.37%) | 90.63% (73.83% − 97.55%) | 100% (91.11% − 100%) | 98.04% (88.21% − 99.9%) |
| E. coli         | 0% (0% − 32.14%)     | 100% (67.85% − 100%)    | 100% (93.69% − 100%) | 98.61% (91.46% − 99.93%) |

**Discussion**

Pathogen identification is crucial in VAP diagnosis and treatment, building a time-saving method friendly to clinical department is the key point to reduce severe and death rate. Nanopore technology had been used in several epidemiological diagnosis occasions [12, 16–22]. However, there were no unified procedure about how to deal with the respiratory samples, and the efficiency of different methods to different pathogens is not clear. Here we compared different methods, and provided a theoretical basis for the choice of methodology.

This study included 83 ETA samples from patients with suspected VAP who had been intubated for more than 48hrs. Like previously indicated by other researchers [17, 23], our pilot also found that DNA extracted directly from ETA were dominated by host genome, leading to a poor sequencing performance with no pathogen detected. Therefore, the removal of human genome has become a necessary measure of sample processing. Human genomes were removed from all 12 samples after depletion, and the genomic abundance of pathogens and sequence time was significantly improved after depletion, suggesting that the depletion operation of saponins was of great significance for the optimization of sequencing results.

However, there is no clear conclusion on the effect of saponin depletion on the pathogen genome. After comparing the effects of depletion on the 7 ATCC pathogens involved in this study, the results showed that depletion did not significantly affect the abundance of 6 pathogens except *S.pneumoniae*. This result is similar to the research result of Charalampous et al. from the UK, which may be due to the simultaneous lysis of *S.pneumoniae* genes during the process of lysis of human genome [17, 24]. In this study, however, among 83 cases, 7 cases involving *S.pneumoniae* (S14, S16, S23, S36, S68, S78, S80) showed that the clinical culture were negative and sequencing results were positive, and 6 samples besides S16 also suggest the existence of *S.pneumoniae* from qRT-PCR results. Although DNA extraction from human sources may damage pathogen, as *S.pneumoniae* is difficult to be
cultured in clinical practice and has a low positive rate, sequencing is still a better choice for detection of *S.pneumoniae*.

Whether to conduct PCR amplification during library build process is also one of the issues that needed to be discussed. One of the advantages of Nanopore sequencing is that the DNA sequence information can be obtained without PCR amplification, thus preserving the methylation and other modification information on the DNA, which is conducive to further data mining and processing. In this study, the differences in pathogen detection between PCR amplification and non-PCR amplification in 29 samples were compared, and for most pathogens, adequate and effective pathogen information could still be obtained without PCR amplification and sequencing.

Some samples showed positive from culture and qRT-PCR results but negative from sequencing results (*K.pneumoniae*: S11, S23; *S.aureus*: S27; *Paeruginosa*: S40, S55, S63; *S.maltophilia*: S59; *A.baumannii*: S40, S44, S45), which may due to the high content of human or oropharyngeal pathogen genome content who covered up the pathogen information. Some samples showed positive from clinical culture results but no positive results from sequencing and qRT-PCR (*K.pneumoniae*: S43; *A.baumannii*: S44, S45), which may due to sampling error. Repeated sampling, proper protection and cleaning of oropharynx during sampling, and adequate saponin mixing of samples can appropriately avoid the occurrence of such phenomena.

For patients with tracheal intubation for 48hrs or more, the types of pathogens in the lower respiratory tract decreased with the extension of intubation time, but the abundance of individual pathogens increased with the extension of intubation time [25]. For patients with newly intubated trachea and suspected VAP, the sequencing results often present a mixed form of multiple pathogens. In addition, the number of reads of different pathogens may vary greatly in the sequencing results of the same sample. For such samples, it is still necessary for clinicians to judge the specific pathogenic bacteria types and precise drug use in combination with the clinical manifestations of patients.

**Abbreviations**
Abbreviations | Full name
--- | ---
VAP | ventilator associated pneumonia
ETA | endotracheal aspirate
MV | mechanical ventilation
PUTH | Peking University Third Hospital
NF-Water | Nuclear-Free-Water
*Paeruginosa* | *Pseudomonas aeruginosa*
*A.baumannii* | *Acinetobacter baumannii*
*K.pneumoniae* | *Klebsiella pneumoniae*
*S.aureus* | *Staphylococcus aureus*
*S.maltophilia* | *Stenotrophomonas maltophilia*
*S.pneumoniae* | *Streptococcus pneumoniae*
*E.coli* | *Escherichia coli*

**Declarations**

**Ethics approval and consent to participate**

This study was proved by Ethics Committee of Peking University Health Sciences (IRB00001052) and the Ethics Committee of Peking University Third Hospital (M20200352).

**Consent for publication**

All presentations of case reports have got consent.

**Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

Authors declared no conflict of interest exists.

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**Authors’ contributions**

Nan W had access to the result in the study and takes responsibility for results. Ning S, Robert PD, Ence Y, Bei H and Nan W contributed to the research idea and design. Beibei L, Lina S, Wei Y and Shining B chose the patients.
and made the clinical diagnosis. Nan W and John R. ED built the sample collection and DNA extraction protocol. Nan W, Chenxia G, Meng W, Wenting W and Jianing W did the ETA collection, and Nan W, Ping Y, Lin Y, Qiaoshan T performed the ETA management. Nan W extracted sample DNA and sequencing. Nan W, Piyush R, Changyu T, Chao L and John R. ED contributed to bioinformatic analysis. Jiajia Z and Nan W contributed to clinical pathogen identification. Nan W contributed to data analysis. Nan W, Robert PD, Ning S and Bei H wrote the manuscript. All authors have reviewed and approved the final manuscript for submission.

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Figures
Sample Collection and Processing

105 patients over 18 years old with endotracheal intubation more than 48 hours

- No clinical results available or sample not enough → 16 patients
- 89 samples extracted DNA

- No DNA extracted → 6 patients
- 83 samples

- Host DNA depletion
  - Yes → 83 DNA samples
    - PCR → 22 samples
    - nonPCR → 83 samples
  - No → 12 DNA samples
    - qRT-PCR → 12 samples
    - Sequencing → 3 samples

Figure 1

Flow Chart 1
Endotracheal Aspirate (ETA) Host DNA Depletion

4x volume PBS added into ETA samples
Separate into 1ml each
Centrifuge and collect sediment
250μl PBS resuspend

Undepleted

200μl NF-Water

Room temperature place for 10min
350μl NF-Water

12μl NF-Water

8000rpm centrifuge for 5min and collect sediment
100μl PBS resuspend

110μl PBS

37°C water bath and shake for 15min
8000rpm centrifuge for 5min and discard supernate
800μl PBS resuspend
Centrifuge and discard supernate
1000μl PBS resuspend
Centrifuge and collect sediment for further research

Depleted

200μl 5% saponin

12μl 5M NaCl

100μl HL-SAN Buffer
10μl HL-SAN

Figure 2

Flow Chart 2
Metagenomic Sequencing

nonPCR

Library preparation
SQK-RAD004/SQK-RBK004

Sequencing with MinION
(Flow cell: R9.4)

PCR

Library preparation
SQK-RPB004

Data process with MinKNOW (v.19.12.5)
Basecalling with Guppy (v.3.2.10)

Raw data flit with Nanofilt (v.2.7.1)
Summary with Nanoplot (v.1.32.1)

Human reads removed with minimap2 (v.2.17) and Samtools (v.1.11)
Pathogen identified with kraken2 (v.2.1.1)

Figure 3
Flow Chart 3
Human ETA metagenomic analysis process

Figure 4

Flow Chart 4
Figure 5

Effect of Host DNA Depletion. Depleted VS Undepleted, *: P<0.05; **: P<0.01; #: P<0.001

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalFile1Primer.pdf
- AdditionalFile2ResultsComparison.pdf
- AdditionalFile3ComparisonofPCRsequencingandnon.pdf