Rapid ultrasensitive diagnosis of pneumonia caused by Acinetobacter baumannii using a combination of enrichment and phage-based qPCR assay

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Abstract
Background Accurate and rapid identification of ventilator associated or nosocomial pneumonia caused by Acinetobacter baumannii (A. baumannii) could improve the treatment.

Methods In current study, we developed a phage-based real-time quantitative PCR (qPCR) combined with enrichment culture for rapid and specific detection of viable A. baumannii in sputum from lung infections. Through short-term plate incubation, bacteria can be enriched and the DNA polymerase reaction disturbance of sputum can be decreased greatly. This approach is based on detecting phage replication in live A. baumannii cells through Taqman qPCR.

Results Through the built detection system, down to 1 CFU of A. baumannii can be detected within 6 h in spiked sputum samples without any steps of bacteria isolation and DNA extraction. The established method was then applied to detecting both A. baumannii in simulated sputum with 100% agreement with the spiked amount of the bacteria and one clinical sputum sample from an 80-year-old male lung infection patient caused by A. baumannii with perfect accuracy, demonstrating that the assay developed in this study has the merits of high rapidity, high sensitivity, good specificity and being able to detect live bacteria not dead bacteria.

Conclusions The assay is a potentially clinical method for diagnosis of bacterial pneumonia infection caused by A. baumannii or other bacterial infection in sputum or complicated samples through switching to other types of phages.

Background
Pneumonia and lung infections caused by multidrug resistant bacteria (MDR) such as Acinetobacter baumannii (A. baumannii), is the most common healthcare-acquired infections among the critical illness with significant morbidity and mortality.[1, 2] Without appropriate initial antibiotic therapy, the mortality rate of community-acquired pneumonia caused by A. baumannii has been reported to be as high as 64%. [1] Accurate and rapid diagnosis of bacterial pathogens in pneumonia is pivotal for the guidance of medication and reducing mortality.

Diagnosis of the bacteria in lung infections is traditionally based on cultures and serology.[3-5] The methods of culture depend on culture and isolation of target bacteria and are the current "gold
standard” for the diagnoses and identifications of bacterial pathogens. While, the culture method is time-consuming, usually offering delayed results and seriously retarding the clinically choosing medicine and effective and timely treatments.[6] Serology based methods require a second convalescent-phase sample, which limits the clinical usefulness of these techniques.[6] Furthermore, the sensitivity of cultures is poor and false negative rate is usually high. Nucleic acid amplification technique are another common-used means, including conventional PCR and real-time quantitative PCR (qPCR), which usually amplify some specific genes of bacteria and are considered faster, more sensitive, and more specific than traditional cultures and serology.[7-9] Nevertheless, due to the high sensitivity and easy-to-contaminate of PCR of bacteria based techniques, the false positive results tend to happen.[10] Especially, the PCR of bacteria based methodologies cannot discriminate viable bacteria from dead bacteria as well as are inhibited by interference factor that existing in sputum specimen from pneumonia infection.[11, 12]

Bacteriophages or phages are viruses that specifically infect host bacteria. After a lytic phage binds to and infects its host, a single bacterial cell can produce hundreds of progeny phages in a few minutes. Through facile procedures, it is easy to isolate from water or soil samples in the environment and obtain a large amount by co-culturing with the host. Bacteriophages have been developed for various assays, such as the detection of Mycobacterium tuberculosis in sputum.[13-17] Conventional methodologies based on phage for the recognition of bacteria are focused on counting the formed plaques by the double-layer plate culture after the infection of phage to the bacteria, where it takes two to three days to get the results.[18] In recent decades, increasing methods relying on the high affinity of the bacteriophages to the host bacteria have been developed for the detection of pathogenic bacteria.[19-22] In addition, the released components after lysis by phages can be determined to develop assays for the detection of bacteria, for example, β-galactosidase or progeny phages.[23, 24] Furthermore, A SYBR green qPCR had been built for phage-based detections of bacteria, determine 1 CFU/mL within 72 h, which cannot satisfy the instant demand clinically.[25] The samples involved in these above mentioned methodologies mostly contained simple matrix such as milk, which is in favor for culturing the host bacteria and thus contributes for the detection through
phages, while in the complex samples such as serum or sputum, phages might not infect the host well, so it is a challenge to develop a phage-based methodology, especially in the samples of sputum. Recently, we reported a phage-based qPCR methodology for sensitive diagnosis of bloodstream infection, where a platform was built to detect A. baumannii (down to 10 CFU in 100 µL serum) in serums within 4 h without bacteria isolation and DNA extraction.[26] However, sputum samples are more complex than serum samples and sputum disturbs the PCR reaction seriously so as to be not able to detect targets in sputum directly by qPCR.

To develop a method to detect bacterial pathogens in Pneumonia sensitively, rapidly and accurately, in this study we exploited a phage-based method combining quantitative PCR (qPCR) with a short-term plate culture for the rapid, sensitive and noninvasive detection of viable A. baumannii in sputum samples from a patient with lung infection. As depicted in Fig. 1., after the sputum samples spread on culture plates were incubated for 2 h, the bacteria on the plates were collected for co-culturing with phage for 3 h, and then the mixture of the phage and the bacteria was subject to qPCR detection of the phage. The Ct changes between the time point 0 h and 3 indicated whether there were A. baumannii or not in sputum samples. Through the system, down to 1 CFU in 100 µL sputum samples was able to been detected by the established detection system within 6 h. Through spiking A. baumannii and several other common bacterial pathogens in sputum, the specificity of the system has been proved. Moreover, the established system has been applied to a clinical sputum sample to validate the system clinically and a satisfactory result has been obtained. The developed methodology here is potentially used to diagnose pneumonia caused by A. baumannii from sputum samples directly.

Methods

**Bacterial strains and culture condition**

All bacterial strains used here are listed in tab. 1. The phage p53 was isolated previously, with a host of A. baumannii strain LB8, an isolated strain from a clinical pulmonary effusion sample. All strains in this study were routinely streaked onto a Luria Bertani (LB) plate. And then the plates were incubated overnight at 37 °C. After picking a single colony on the plate into 5 mL LB broth, the bacteria were
grown overnight at 37 °C and 200 rpm. Then the bacteria were spread onto LB agar plates for 12 h and collected by cotton swabs. Bacteria were released by stirring cotton swabs in 1 mL LB broth. Then the bacterial suspension was centrifuged at 6000 rpm for 5 min. After discarding the supernatant, the bacterial precipitation was re-suspended in 1 mL fresh LB broth. The OD$_{600}$ of the bacteria solutions was adjusted to 0.45, which is corresponding to about 6x10$^8$ CFU/mL of the bacteria, which had been calculated by plate dilution as shown in Fig. 2 before use. After the determination of the concentration of the bacteria in CFU/mL, serial dilutions of the bacteria were adopted for the experiments. Unless specified, all strains listed in tab. 1 were treated by the same protocol before use.

Tab. 1. The information of the bacteria used in this study
| Strain               | Strain number   |
|---------------------|-----------------|
| A. baumannii 3304   | Clinical isolate|
| A. baumannii 2703   | Clinical isolate|
| A. baumannii 3679   | Clinical isolate|
| A. baumannii 3064   | Clinical isolate|
| A. baumannii 3782   | Clinical isolate|
| A. baumannii 3431   | Clinical isolate|
| A. baumannii 5761   | Clinical isolate|
| A. baumannii 3902   | Clinical isolate|
| A. baumannii 3951   | Clinical isolate|
| A. baumannii 3362   | Clinical isolate|
| A. baumannii 3682   | Clinical isolate|
| A. baumannii 3045   | Clinical isolate|
| A. baumannii 2140   | Clinical isolate|
| A. baumannii 3305   | Clinical isolate|
| E. coli             | ATCC 25922      |
| S. aureus           | N315            |
| P. aeruginosa 3524  | Clinical isolate|
| S. pyogenes         | ATCC 12344      |

**Preparation of the phage p53 solution**

The phage p53 against the host *A. baumannii* strain, *LB8*, was isolated from a sewage sample in the
The sequence of p53 was determined in the lab previously. The preparation of phages p53 and determination of phage titers are based on our previous publication[26]. Briefly, the purification and PFU determination of the phage were realized by double-layer plate techniques. Through put 100 μL purified phage one a double layer plate with LB8 lawn in a soft-agar layer on a regular LB agar layer and incubated overnight at 37 °C, the soft-agar layer was collected in 5 mL phage buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 2 mM CaCl₂.) Tris base was bought from Sigma-Aldrich, Co, St. Louis, MO, USA. After filtered by sterile filter, the phage solution was obtained. Single clear plaques of a serial dilution of the phage solution were used for determination the concentration of a phage solution in PFU/mL. The PFU-determined phage solution was stored at 4 °C for further use.

qPCR assay

The primers and probes were designed for specifically detecting p53, as described previously. [26] The sequences of the primers and the probe were: forward, 5'- CGGATGTGCAATATTAC-3'; reverse, 5'- TTCCCATTGGCGATTGGT-3'; Probe, 5'- FAM- ATTGATGTGCGACACCTGC -BHQ1-3'. The primers and probes were designed for detecting gltA gene specific to A. baumannii.[27] The sequences of the primers and the probe for gltA gene were: forward, 5'- CGTCTCTATCACAACAA-3'; reverse, 5'- GCTGACCTACAGTATATTTG-3'; Probe, 5'-FAM-CTTGACATTGAA CACATCAACCACCG-BHQ1-3'. All the primers and probes were synthesized in Shanghai Sangon Co., Ltd. (Shanghai, People’s Republic of China). 2xqPCR reaction mixture was prepared by mixing 0.5 μL Taq polymerase (activity unit 2.5 U/μL, expressed and purified in the laboratory), 1.6 μL dNTP (2.5 mM for each, from Takara Biomedical Technology (Beijing) Co., Ltd, China), milliQ water 5.9 μL, 2 μL 10xbuffer (200 mM Tris-HCl pH 8.3, 200 mM KCl, 100 mM (NH₄)₂SO₄, 20 mM MgSO₄ and 5% NONIDET P-40 SUBSTITUTE from AMRESCO Inc., Radnor, PA, USA). Each qPCR reaction system was made of 10 μL 2xqPCR reaction mixture, 0.4 μM of the probe, 0.4 μM of the primers, 2 μL of the template and water added to total volume of 20 μL, for the detection of p53. The qPCR amplification was performed on CFX96, Bio-Rad Laboratories, Hercules, CA, USA. The procedure was set as: 95 °C for 3 min, 40 cycles of 95 °C for 5 s and 60 °C for 1 min, and the fluorescent signal was acquired at the step of 60 °C.
Optimization of phage concentration and the incubation time of p53 with LB8 for the detection of A. baumannii

After incubation at 37 °C for 8 h, the bacteria on LB agar plates were collected by cotton swabs in 1 mL LB broth. Through serial dilution of the bacteria, different concentrations of LB8 (10^0, 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 CFU/mL) in LB broth were obtained according to the description above. 500 µL LB8 in LB broth were mixed with 500 µL of p53 at the concentrations of 10^2, 10^3, 10^4, 10^5, 10^6 PFU/mL, respectively. 50 µL of the mixture of LB8 and p53 was immediately taken out and stored at -20 °C, which was set as 0 min. Next, the mixture of LB8 and p53 were incubated at 37 °C with shaking at 200 rpm. Then 50 µL of the mixture was taken out every 50 min. All the samples were stored at -20 °C immediately after taken out for the qPCR detection of p53 after sampling was completed.

Optimization of the plate culture time for the detection of A. baumannii

The culture time of LB8 spiked sputum samples on LB agar plates was optimized for the detection platform. Before spiking the bacteria, the pure sputum samples were investigated by culture and plaque assays for the absence of either LB8 or p53. The bacteria of serial 10-fold dilutions of LB8, was mixed with the pure sputum to the final concentrations of LB8 with of 10^0, 10^1, 10^2, 10^3, 10^4, 10^5 and 10^6 CFU/mL. Then 100 µL of LB8 spiked sputum was transferred onto LB agar plates containing three glass beads and spread by moving the glass beads on the plates. The plates were incubated at 37 °C. And one plate was taken out at the time points of 0 h, 2 h, 4 h, 6 h, 8 h and 10 h, respectively. Substances including bacteria on the plates were collected by cotton swabs and released by stirring cotton swabs in 1 mL LB broth. Then the bacterial suspension was centrifuged at 6000 rpm for 5 min, discard the supernatant and bacterial precipitation was re-suspended in 1 mL fresh LB broth. Afterward, 500 µL bacteria in LB broth were mixed with 500 µL 10^3 to 10^4 PFU/mL p53 in phage buffer. The other 500 µL bacteria were for qPCR detection of gltA gene of A. baumannii. After the mixture was cultured for 180 min, 50 µL of the mixture was taken out for qPCR detection of p53. The experiments have been run for three times.
**qPCR detection of gltA gene of A. baumannii**

The other 500 µL bacteria were extracted by a commercial bacterial extract kit (Omega Bacterial DNA Kit) based on silica-column principle. The isolated DNA was for qPCR detection of gltA gene of *A. baumannii*. The experiments have been performed for three times.

**Assay performance with simulated clinical sputum samples**

15 bacteria-spiked sputum samples were prepared as listed in tab. 2., including 14 sputum samples spiked by 14 different clinical *A. baumannii* strains in three different concentrations ($10^1$~$10^2$, $10^3$~$10^4$, $10^5$~$10^6$ CFU/mL) mixed with the other four bacteria (*E. coli*, *S. aureus*, *P. aeruginosa* and *S. pyogenes*, $10^5$ CFU/mL to $10^6$ CFU/mL for each), and 1 sample of the mixture of the four non-*A. baumannii* bacteria with three different concentrations ($10^1$~$10^2$, $10^3$~$10^4$, $10^5$~$10^6$ CFU/mL for each). The plate was taken out at time points of 0 h and 2 h. The following steps were done according to the description above. The mixture of phage and bacteria was detected by qPCR to detect p53. The Ct difference at time points of 0 h and 2 h were obtained for analysis. All of the collected samples were tested in triplicate by qPCR.

**Apply the method to diagnose an 80-year-old male lung infection patient**

To evaluate the practicability for detection of *A. baumannii*, the established method has been used for analyzing a real clinical sputum sample from an 80-year-old male lung infection patient presented with fever, dyspnea, and cough 3 weeks after his last examination. Sputum collected from broncho-alveolar lavage fluid of the patient was detected by the method. And a regular culture method also has been used for analyzing the sample. The traditional culture method was realized through spreading 100 µL of the sputum on LB agar plates with 3 glass beads, and cultured 16 h to get bacterial colonies. Through amplifying 16S rDNA gene using the commonly used primers 27F (5’-AGAGTTTGATCTGGCTCAG-3’) and 1492R (5’-GGTTACCTTGGTACGACTT-3’), the PCR products were subjected to sequencing by Shanghai Sangon Co., Ltd. (Shanghai, People’s Republic of China). And through blast the species of the bacteria were determined.

**qPCR data analysis**
There are two points should be satisfied when the sample contains *A. baumannii*. First, the Ct value at a time point should be smaller than the Ct value at the culture time 0 min, indicating that the phage are amplified at that time point and the sample might contain *A. baumannii*. Second, in consideration that the acceptable Ct value deviation of qPCR detection is normally lower than 0.5, 0.5 is a threshold for the change of Ct. If the Ct changes (ΔCt) between the initial and the other time point of the sputum sample is above 0.5, the sample contains *A. baumannii*. Otherwise, there was no *A. baumannii* in the sample.

**Results**

Before exhibiting the results in this study, several results and conclusions should be shared from our previous publication. [26] 1. As determined by regular PCR and the agarose gel analysis in the publication, the set of the primers/probe was specific to p53 without any interference from different strains of *A. baumannii* as well as other four common bacteria (*E. coli*, *S. aureus*, *P. aeruginosa*, *S. pyogene*). 2. The burst time for p53 was determined as 50 min by qPCR. As well, the phage genome was released well through initial heating of 95 °C for 3 min from the amplification efficiency. 3. The detection limit of p53 in phage buffer was $10^2$ PFU/mL, so for the following optimizations of the p53 concentration for the detection of *A. baumannii*, the concentration of phage p53 started from $10^2$ PFU/mL. However, sputum has more profound inhibition to qPCR than serum, which is the matrix in the previous study. [26] We could not get any Ct values for any concentration of p53 in sputum by direct qPCR. Therefore, we changed the strategy to decrease the interference of sputum through short culture on LB agar plates. Therefore, here we optimized the phage concentration by the new strategy.

Optimization of phage concentration and incubation time of p53 with LB8 for the detection of *A. baumannii*

Through optimization of phage concentration, the sensitivity and the concentration range of the detection for the host bacterial pathogen can be improved. The bacteria were scraped from culture plates, where the bacteria were different from the ones cultured in broth. The optimization of the concentration of p53 was started from $10^2$ PFU/mL since the lowest concentration of $10^2$ PFU/mL can
be detected. [26] Serial 10-fold from $10^2$ to $10^6$ PFU/mL of the concentration of p53 (totally 5 concentrations of $10^2$, $10^3$, $10^4$, $10^5$, $10^6$) were adopted. And at each concentration of p53, the host concentrations were variated from $10^0$ to $10^6$ CFU/mL, including all possible CFUs of the bacteria in sputum samples. The sample were collected every 50 min for the detection of qPCR until 250 min and Ct values were recorded. The plots of Ct values vs. the culture time of the mixture of p53 and LB8 in LB broth were illustrated in Fig. 3. When the concentration of p53 was higher than $10^4$ PFU/mL, an increasing detection range of the bacteria concentration and sensitivity of the bacteria can be obtained as shown in Fig. 3C-E, while at low concentration of p53 ($10^2$ PFU/mL) the sensitivity became low, as indicated in Fig. 3A. Therefore, when the concentration of phage p53 was $10^3$ to $10^4$ PFU/mL as revealed in Fig. 3B and 3C, the sensitivity was the highest and the detected range of the bacteria is the broadest, where 100 CFU/mL of A. baumannii was detected at the time point of 100 min. Therefore $10^3$ to $10^4$ PFU/mL of phage p53 was selected for the following detection of bacteria in sputum samples. In addition, longer incubation time will lead to higher sensitivity. For example, at the culture time of 200 min, the detection limit arrived at 10 CFU/mL with obvious signal (Ct changes) as shown in Fig. 3B and 3C. Consider the sensitivity and the rapidity of the detection, the culture time of the phage and the bacteria was set as 180 min.

Optimization of the plate culture time for the detection of A. baumannii

In order to optimize the plate culture time to detect a broad concentration range of the host bacteria, the concentrations of LB8 were spiked in sputum to get the bacteria concentration ranged from $10^0$ CFU/mL to $10^6$ CFU/mL. And then applied the LB8 spiked sputum on LB agar plates and culture for 0, 2, 4, 6, 8 and 10 h. The plots of Ct values vs. the culture time of the mixture of p53 and LB8 in LB broth at the time interval 2 h were illustrated in Fig. 4A. The results have shown that when the culture time was 2 to 4 h, the detection system showed an increasing detection range and sensitivity of the bacteria as shown in Fig. 4A, while with too low culture time the sensitivity became low. Therefore, at the culture time of 2 h and 4 h (in Fig. 4A) $10^1$ CFU/ mL A. baumannii was able to be detected easily,
where ΔCt is much higher than the cut-off value 0.5. At the culture time of more than 4 h, $10^1$ CFU/mL of the host bacteria can be also detected, but less ΔCt values were obtained for the detection of different concentrations of the bacteria than those at 2 h or 4 h. When running the experiments, we found that at long culture time, the things on the plate became sticky, probably resulted from other bacteria or easy-to-grow organisms, which might inhibit the infection of phage to the bacteria. This might be the reason why at long culture time, less difference of phage numbers arose. Furthermore, less time is better for rapid detection. Therefore, 2 h was used for the following detection of bacteria in sputum samples.

At the same time, the established method was compare with the traditional qPCR method for amplifying gltA gene of A. baumannii. The bacteria were scraped from the plates applied with sputum samples and subjected to DNA extraction by kit, followed by qPCR for the bacteria. Here a commercial kit for extracting genome DNA of A. baumannii, because the substances scraped from the culture plates still interfere directly detecting the specific gene of A. baumannii by qPCR. The corresponding results are revealed in Fig. 4B. At the culture time of 2 h, the detection limit is about $10^4$ CFU/mL. For detecting gltA gene of A. baumannii, the limit of detection could reach $10^1$ CFU/mL but culture time was about 6 h, which takes total about 9 h (6 h for plate culture and 3 h for DNA extraction and qPCR detection) for the whole detection from the sputum to final results.

**Assay performance with simulated clinical sputum samples**

*L8* in Logarithmic-phase was spiked to the sputum to the final bacteria concentrations of $10^0$ CFU/mL to $10^6$ CFU/mL. The p53 concentration of $10^3$ to $10^4$ PFU/mL was used as optimized previously. [26] 15 simulated sputum samples, including 14 clinical A. baumannii strains mixed with the other four common bacteria, which were *E. coli*, *S. aureus*, *P. aeruginosa*, *S. pyogene*, 1 sample with the mixture of the four non-A. baumannii bacteria, were designed to test feasibility of the phage-based platform to detect other A. baumannii strains and evaluate the specificity of the method. The detection results by the developed method as well as the spiked CFU of the bacteria for the 15 simulated clinical sputum samples have shown in Tab. 2. The phage-based qPCR method displayed
consistent results with the traditional culture method. By the developed method, down to $10^1$ CFU/mL of *A. baumannii* can be detected well in 6 h with a high sensitivity. Here 100 µL sputum was taken for the detection, which means 1 CFU can be probed by the current methodology.

Tab. 2. Detection results of 15 simulated sputum samples
| Strain Number | Plaque | $10^5$–$10^6$CFU/mL | Detectable or not (Y/N) | $10^3$–$10^4$CFU/mL | Detectable or not (Y/N) | $10^1$–$10^2$CFU/mL | Detectable or not (Y/N) |
|---------------|--------|----------------------|------------------------|----------------------|------------------------|----------------------|------------------------|
| 3304          | +++    | $18.41\pm0.32$       | Y                      | $9.35\pm0.29$        | Y                      | $2.18\pm0.0$         |                       |
| 2703          | +++    | $11.23\pm0.1$        | Y                      | $10.82\pm1.3$        | Y                      | $5.65\pm1$           |                       |
| 3679          | +++    | $11.67\pm1.23$       | Y                      | $7.62\pm0.73$        | Y                      | $1.02\pm0$           |                       |
| 3064          | +++    | $14.62\pm0.11$       | Y                      | $4.6\pm0.3$          | Y                      | $1.31\pm0$           |                       |
| 3782          | ++     | $7.57\pm2.05$        | Y                      | $1.83\pm0.44$        | Y                      | $0.72\pm0$           |                       |
| 3431          | ++     | $7.1\pm1.09$         | Y                      | $6.01\pm2.01$        | Y                      | $3.22\pm0$           |                       |
| 5761          | +++    | $11.62\pm0.65$       | Y                      | $7.38\pm0.15$        | Y                      | $2.21\pm0$           |                       |
| 3902          | +      | $5.24\pm0.29$        | Y                      | $1.12\pm0.18$        | Y                      | $0.62\pm0$           |                       |
| 3951          | +++    | $16\pm1.77$          | Y                      | $11.22\pm0.89$       | Y                      | $7.32\pm2$           |                       |
| 3362          | +++    | $16.02\pm0.18$       | Y                      | $18.53\pm2.06$       | Y                      | $6.28\pm1$           |                       |
| 3682          | +++    | $17.33\pm0.57$       | Y                      | $2.62\pm1.04$        | Y                      | $3.97\pm0$           |                       |
| 3045          | +++    | $11.08\pm0.38$       | Y                      | $6.58\pm1.02$        | Y                      | $1.12\pm0$           |                       |
| Mixture of E. coli, S. aureus, P. aeruginosa, S. pyogene | -     | $0.48\pm0.28$        | N                      | $0.04\pm0.01$        | N                      | $0.32\pm0$           |                       |

+++ and ++ mean the plaque of the phage on the tested bacteria is very clear, clear and faint.
of the plaques, respectively. --- means no lysis plaque.

**The performance of the method in diagnosing an 80-year-old male lung infection patient**

To evaluate the performance of this method, the developed method as well as a regular culture method has been used for detecting *A. baumannii* in a real clinical sputum sample from a patient. The regular culture method was realized through isolating the bacteria from the sputum sample and identifying the bacteria through 16S rDNA PCR and sequencing. As shown in Fig. 5., the results showed that the method could effectively detect bacteria in a clinical sample, the result of which was consistent with the traditional methods (the data of the traditional methods were not shown here).

**Discussion**

Nosocomial lung infections are critical causes of morbidity and mortality worldwide, requiring early diagnosis and treatment. Herein, a phage-based qPCR method integrated with a short-term plate culture for rapid diagnosing viable *A. baumannii* in sputum was established to be applied for diagnosis of the bacterial lung infections. The built assay was based on the Taqman qPCR for monitoring the phage amplified by the host to detect the host pathogen causing lung infections. The new method in this study offered a suitable method for the rapid and specific detection of pathogenic bacteria in clinical samples with short enrichment cultivation, delivering additional information concerning bacteria viability. Therefore, we evaluated the practicability of the method for the rapid identification of microorganisms in specimens of patients with lung infections. The sequence alignment between the genome sequences of the phage and the host bacteria could eliminate the nonspecific amplification signal from the host bacteria. Further, through blast analysis of the amplified p53 sequence, there is only one Acinetobacter phage with high coverage and high similarity to the amplified sequence of p53 and the coverage percentages for the other sequences of the blast results are low. Furthermore, in many previous reports, accurate quantification of phages by PCR requires efficient removal of intact bacterial cells from the samples and incorporation of effective DNase treatment step, while the established method in this study simplified the process. The assay in this
study can be completed in 6 h from the sputum to the final results, which can satisfy the requirement of rapid diagnosis. By the current developed assay, 1 CFU of the host bacteria in 100 µL sputum samples can be detected through phage amplification. The detection limit was greatly improved through three amplification steps including short-term plate incubation, phage amplification and the target sequence amplification.

Further, due to the initial short-term plate incubation of the sputum samples, the bacteria could be amplified and high matrix interference could be decreased. Through 2 h co-culture of the phage with the host bacteria, one bacterium may produce hundreds of prophages. The developed assay was able to be completed in 6 h with a detection limit of 1 CFU of the target bacteria even in the presence of high concentrations of interference bacteria. Since phages propagate only through viable bacteria, the method allows the identification of live bacteria providing more additional, important and clinically and epidemiologically relevant information than the assays based on target bacterial DNA. Especially for patients taking medicine, the characteristics of this method can help patients adjust the dosage in time. Another advantage of the method is not dependent on DNA extraction and purification. If the detection of genes of the target bacteria is combined with the short-term plate culture, the matrix in the sputum samples still interferes with the detection. While integrated with the short-term plate culture, the phage-based qPCR method can directly detect the gene for the phage without DNA extraction, due to mixing the same volume of phage solution with the host bacteria to dilute the inhibiting components as well as the burst of the phage resulting in amplifying the target and promoting the sensitivity of the detection.

Furthermore, the practicability and the specificity of the method was demonstrated by detecting the mixture of the target bacteria with other common bacterial pathogens in lung infections, including E. coli, S. aureus, P. aeruginosa and S. pyogene, spiked in sputum samples of lung infection patients. Compared with the method based on the conventional microbiological culture, the specificity of the developed method was 100%. The detection limit of this method was 1 CFU in 100 µL sputum. Otherwise, the method can be applied for antibiotics-resistant detection through directly culturing the sputum samples with antibiotics for 1 or 2 hours,[28, 29] and then the amount of the bacteria will be
different for drug-sensitive and drug-resistant bacteria. Otherwise, there cases of pulmonary infection based on phage therapy are increasing[30, 31] and this method provides ideas for phage screening and monitoring in phage therapy.

However, there are still some questions to resolve for the developed method for clinical application. A common problem based on phage for detecting host bacteria is the host spectra of the phages. Up to now, there have been no phages discovered infecting all strains of a species, which might be solved by engineered phage. Therefore, the developed method in this study can only detect p53-sensitive A. baumannii strains, not all A. baumannii. Circumventing this problem, we can combine a phage cocktail and multiplex-qPCR to detect more bacterial strains and species in sputum samples. Owing to the resource limitation, the real clinical patient specimens were small and we hope to evaluate the clinical application prospect of this method more accurately through more actual patient samples in future.

Conclusion
Here, a phage-based qPCR methodology combined with enrichment culture through a short-term plate culture on culture plates for the determination of A. baumannii in sputum samples was established and validated through a series of experiments. The assay could probe as low as 1 CFU in 100 µL sputum within 6 h, without any steps of bacteria isolation and DNA purification. The possibility of the rapid detection of pathogenic bacteria applied in clinical samples of lung infection patients will help to improve diagnosis of microbiological infections and clinical management of these patients. The method is applicable for clinical application in diagnosing bacteria causing lung infections.

Declarations

Acknowledgements
Not applicable.

Ethics approval and consent to participate
Compliance with ethical standards.

Consent for publication
Not applicable.
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

The authors declare that they have no competing interests.

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Author Contribution

J.P.Y., and H.P.W., conceived the study; J.L., and J.P.Y., designed and performed the experiments; J.L., and J.P.Y., analyzed the data; M.W.J., J.H.L., and J.X., contributed materials and provided advice; J.L., and J.P.Y., wrote-reviewed and edited the paper.

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Figure 1

Schematic diagnosis procedure of the proposed method for the rapid and ultra-sensitive detection of viable A. baumannii in sputum samples from patients combing a short-term plate culture with phage-based qPCR (not to scale).
Figure 2

The correlation between bacteria concentration logarithm of A. baumannii in CFU/mL and OD600
Figure 3

Optimize the phage concentration to detect different concentrations of the host A. baumannii LB8 (From 100 CFU/mL to 106 CFU/mL). The Ct values vs. the culture time of the phage p53 mixed with A. baumannii at the phage concentration of A. 10^2 PFU/mL, B. 10^3 PFU/mL, C. 10^4 PFU/mL, D. 10^5 PFU/mL, E. 10^6 PFU/mL. The errors were obtained from three independent experiments.

Figure 4

A. Optimize the time of the plate culture of the sputum samples. B. Detecting A. baumannii scraped from the plates through qPCR for gltA gene of A. baumannii.
The proposed system was applied to detect A. baumannii in a sputum specimen from a clinical patient.