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Development and Evaluation of a Multiplex Quantitative PCR Assay for Detecting Bacteria Associated with Lower Respiratory Tract Infection

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Abstract

Objectives: This study aimed to establish a multiplex quantitative PCR (MQ-PCR) assay for 12 bacterial pathogens of lower respiratory tract infection (LRTI) and evaluate its performance by a cohort consisting of 211 patients with LRTI.

Methods: The study was divided into two stages: a pilot study to establish the methodology, and a clinical validation study to evaluate its performance. In the pilot study, we established the MQ-PCR and analyzed its performance through the limits of detection, repeatability, specificity, and efficiency. In the clinical validation study, we enrolled 211 sputa and/or bronchoalveolar lavage fluid (BALF) to detect pathogen by

MQ-PCR. The MQ-PCR takes only 3 h from the sample obtained to complete pathogen detection.

Results: The limit of detection was 1000 copies/mL, and the efficiency could reach over 95%. When cutoffs of $\geq 10^5$ copies/mL for sputum and $\geq 10^4$ copies/mL for BALF were applied, the sensitivity, specificity, positive and negative predictive values of the MQ-PCR were 77% (95% confidence interval [CI], 67-88%), 94% (95% CI, 93-95%), 25% (95% CI, 19-31%), and 99% (95% CI, 99-100%), respectively.

Conclusions: This study demonstrates that the new MQ-PCR assay is time-saving, more effective and sensitive, and brings us closer to the mainstream adoption of quantitative molecular detection of bacteria.

Keywords: Lower respiratory tract infection, Bacterial pathogens, Bacterial load, MQ-PCR

Introduction

Lower respiratory tract infection (LTRI) is a substantial public-health problem and a leading cause of illness and death in people of all ages worldwide (Collaborators, 2017, World Health Organization, 2020). LRTI also represents a major challenge for medical treatment because of its diverse causes. LRTI caused by different pathogens (viruses and bacteria) may have similar symptoms (Torres et al., 2021), making them difficult to distinguish clinically. When pathogen information is lacking, physicians often use broad-spectrum antibiotics empirically, leading to increased resistance rates (Caliendo

et al., 2013). By some estimates, 30–70% of antibiotic prescriptions for LRTI are inappropriate (Kraus et al., 2017). Therefore, rapid identification and accurate identify pathogens are essential to guide effective treatment and prevention decisions.

The diagnosis of LRTI has greatly advanced in recent years. Many commercial multiplex PCR assays for LRTI-causing viruses have been reported, such as the RespiFinder assay (Reijans et al., 2008) and FilmArray Respiratory Panel (Renaud et al., 2012) et al. However, culture-based remains the standard method for diagnosing respiratory bacteria, being time-consuming (48-72 h) and less sensitive (Chalmers et al., 2011, Driscoll et al., 2017, England, 2014). Although matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) can detect bacterial pathogens in about 0.5-1 h, it can only identify bacteria isolated from culture plates or positive blood culture and patient urine (Hou et al., 2019, Yoon and Jeong, 2021). Thus, a number of recent studies have aimed to develop PCR-based molecular methods to detect bacterial pathogens (Abdeldaim et al., 2010, Gadsby et al., 2015, Greiner et al., 2003, Kais et al., 2006). For bacterial pathogens, quantification of bacteria may be critical to rule out contamination of lower respiratory tract (LRT) specimens with oral commensal flora. Most molecular work done to date has focused on *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* (Abdeldaim et al., 2010, Greiner et al., 2003, Kais et al., 2006), or to merely qualitatively detect pathogens (Collins et al., 2020, Gastli et al., 2021, Jiang et al., 2017). However, a number of studies have focused on quantitative molecular bacterial testing in well-defined community-acquired pneumonia (CAP) set (Gadsby et al., 2015, Gadsby et al., 2016, Johansson et al., 2010,

Werno et al., 2012). The aim of the present study was to establish and evaluate the performance of the multiplex quantitative PCR (MQ-PCR) assay for LRTIs of bacterial causes, allowing for the detection of 12 main bacterial pathogens, including *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Escherichia coli*, *Burkholderia cepacia*, *Staphylococcus aureus*, *Enterococcus faecium*, and *Enterococcus faecalis.*

Materials and methods

Study design

The study was conducted between November 2019 and March 2021 at the National Respiratory Center at the China-Japan Friendship Hospital in China, and was designed in two stages: a pilot study to establish and optimize the MQ-PCR assay and a validation study to evaluate the assay's performance. In the validation stage, we simultaneously ran the traditional culture, next-generation sequencing (NGS), and the MQ-PCR assay on the same respiratory sample to compare the pathogen identification.

Establishment of MQ-PCR assays

The MQ-PCR assay consisted of four separate assays with primer/probe sets covering 12 respiratory bacteria pathogens: assay 1, *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*; assay 2, *P. aeruginosa*, *A. baumannii*, *K. pneumoniae*; assay 3, *E.cloacae*, *E. coli*, *B. cepacia*; assay 4, *S. aureus*, *E. faecium*, *E. faecalis.*

Primers and probes design

We used the National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI-BLAST) to verify candidate oligonucleotide sequence matches to

targets in the GenBank database. To optimize multiplex performance, we modified the assays using Oligo7 and NCBI-BLAST. The NCBI Primer-BLAST was used to verification of optimized oligonucleotide sequences for specificity. To check the sensitivity of species of interest, sequences were also examined against alignments of whole target gene sequences placed in the GenBank database by NCBI-Primer. Using this information, we selected 12 targets for the causative agent test and/or designed for a double-priming oligonucleotide-based MQ-PCR assay for specificity. The sequences of the primers and probes (Cao et al., 2019, Devanga Ragupathi and Veeraraghavan, 2019, Ergin et al., 2009, Fevre et al., 2011, Gadsby et al., 2015, Greiner et al., 2003, Chen et al., 2017, Montazeri et al., 2015, Nomanpour et al., 2011, Nørskov-Lauritsen, 2009, Ozawa et al., 2000) are shown in Table 1.

Control isolates

The positive control for the 12 bacterial strains used in verification assays were as follows: *H. Influenzae* (American Type Culture Collection [ATCC] 9007), *S. pneumoniae* (ATCC 49619), *S. aureus* (ATCC 29213), *K. pneumoniae* (National Collection of Type Cultures [NCTC] 13442), *A. baumannii* (NCTC 13424), *E. coli* (ATCC 29212), *P. aeruginosa* (ATCC 27853), *E. cloacae* (ATCC 45031)， *B. cepacia* (GIMSCAU 1.088)， *E. faecium* (AS 1.130), *E. faecalis* (ATCC 33166), and *M. catarrhalis* (ATCC 25240).Plasmids including the target gene sequences were obtained from Shanghai Shenggong Biological Engineering Co., Ltd. The plasmids was diluted in TE Buffer (Solarbio, Beijing, China) with isometric gradient dilution for use in real-time PCR optimization and as quantification standards. Normal saline was used as the negative control.

Analytical performance

To confirm the specificity of the MQ-PCR assay, clinically isolated bacterial and viral strains (*S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, *E. cloacae*, *E. coli*, *B. cepacia*, *Mycobacterium tuberculosis*, *Legionella pneumophila*, *Bordetella pertussis*, *Mycoplasma pneumoniae*, *S. aureus*, *Staphylococcus epidermidis*, *E. faecium*, *E. faecalis*, *influenza A virus*, *influenza B virus*, *parainfluenza virus*, *respiratory syncytial virus*, *bocavirus*, *metapneumovirus*, *rhinovirus*, *Pneumocystis*, *Aspergillus*, and *Candida albicans*) were detected at a concentration level of 10^6～10^9 PFU (CFU)/mL. The analytical sensitivity was estimated by 20 replications of respiratory tract specimens carrying bacteria at five different concentrations, from 100 to 5000 copies/mL (Supplementary Table 1). The efficiency was measured by detecting target samples at four different concentrations (10^7 copies/mL, 10^6 copies/mL, 10^5 copies/mL, 10^4 copies/mL), drawing a standard curve, and calculating the amplification efficiency. The precision (reproducibility) was evaluated by comparing bacterial load quantification by the MQ-PCR assay for 10～20 replicates for randomly selected engineered bacteria with fragments of *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae* at medium (5×10^4 copies/mL), low (5×10^3 copies/mL), and negative (normal saline).

Verification of the MQ-PCR assay using clinical respiratory samples

Patient selection

Inclusion criteria for the study included the following: adults (aged ≥18 years); patients with clinical or radiographic diagnosis of LRTI, including CAP, hospital-acquired pneumonia (HAP), acute exacerbation of chronic obstructive pulmonary disease

(AECOPD), and bronchiectasis with infection; no antibiotic used within seven days prior to sample collection (limited to outpatients); the patient or their guardian agrees to sign an informed consent form; and collection of qualified sputum specimens (leukocyte > 25/low power field and epithelial cells < 10/low power field by gram-stained sputum smear). Exclusion criteria included pulmonary tuberculosis and cystic fibrosis bronchiectasis. The definitions of CAP (Qu and Cao, 2016), HAP (Shi et al., 2019), AECOPD (Chronic Obstructive Pulmonary Disease Group of Respiratory Society of Chinese Medical Association, 2021), bronchiectasis with infection (Hill et al., 2018) and co-infection are summarized in Supplementary File 1. The enrollment process is shown in Figure 1.

Specimen collection

LRT specimens obtained from clinical microbiology laboratory: LRT samples, including sputum and bronchoalveolar lavage fluid (BALF), were collected in clinical microbiology laboratory. Cases with clinical and radiological evidence of LRTI were identified through checking electronic review. Then the sputum or BALF samples that met the inclusion criteria were subjected to traditional culture.

LRT specimens obtained from emergency and fever clinics: Symptomatic patients were assessed at first presentation. Cases with clinical and radiological evidence of LRTI and without receiving antibiotics within seven days were immediately enrolled, and sputum and urine samples were taken at first presentation. Gram stains on sputum specimens were immediately performed to determine the quality of the specimen and to predict likely pathogens.

Pathogen detection with traditional culture and urine antigen test

Sputum or BALF was cultured and incubated at 35°C in 3-5% CO_2 on MacConkey agar, sheep blood agar, and chocolate agar. Bacteria were isolated from agar by a microbiology laboratory technician then the species were confirmed using MALDI-TOF MS (Bruker Daltonics). *S. pneumoniae* was detected in urine using antigen detection. The remaining sputum or BALF from the culture for MQ-PCR were stored frozen in our laboratories until regular shipment to the Beijing Applied Biological Technologies Co., Ltd, where specimens were stored at −80°C until analysis.

DNA extraction

The Nucleic acid extraction reagents were provided by Beijing Applied Biological Technologies Co., Ltd (Beijing, China). We used a centrifugal column for this study for extraction beginning with fully liquefying the sputum (0.4%NaOH digestion solution). Then, 200 μL of clinical specimen (BALF or sputum) was processed with 15 s of oscillation with 10 μL of proteinase K, 6 μL of Carrier RNA, and 200 μL of enzymatic lysis buffer (Salt and Tris buffer), followed by centrifugation for 2 s and incubation for 5 min at 37°C, followed by addition of 400 μL anhydrous ethanol (Beijing Yili Fine Chemicals Co., Ltd, Beijing, China), then oscillate for 15 s and centrifuge for 5 s, incubation for 5 min at 37°C. We continued processing the sample with 1 min of centrifugation at 12,000 rpm and discarded the wastewater, following with 2 centrifugations of 30 s at 12,000 rpm with 500 μL washing buffer (high salt solution) and rinse (low salt solution), then an additional 3 min at 12,000 rpm to remove the

residual rinsing buffer. A new RNase-free centrifuge tube (1.5 mL) was added to the spin column. We added 100 μL of eluent (Tris buffer) to the middle part of the adsorption film and incubated for 3 min at 37°C, followed by centrifugation at 12,000 rpm for 1 min. The nucleic acid extraction process takes about 1 h.

MQ-PCR conditions

MQ-PCR reactions were performed using an ABI7500 (Applied Biosystems, Waltham MA). The MQ-PCR assay was run in a total reaction volume of 50 μL, including 5 μL oligonucleotide mixture (Primer probes for 12 target pathogens and internal standards), 35 μL nucleic acid amplification reaction solution (Tris-HCl, KCl, Mg^{2+}, Dn(U)TP, thermostable DNA polymerase, UDG enzyme), and 10 μL template. The reaction reagents were produced by Beijing Applied Biological Technologies Co., Ltd (Beijing, China). The cycle parameters for the MQ-PCR were as follows: 50°C for 2 min, 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 55°C for 30 s. It takes about 2 h to complete the MQ-PCR condition. Assay runs was verified using both positive (Inactivated engineered bacteria) and negative (normal saline) controls. The plasmids were diluted in equal proportions (from 1×10^4 copies/mL to 1×10^7 copies/mL), and real-time PCR was performed. Taking the plasmid concentration as the abscissa and the Ct value as the ordinate to draw the standard curve to quantify pathogen. Concentrations were calculated according to the position where the Ct value of the unknown sample overlapped with the standard curve.

Pathogen detection with NGS

We randomly selected 12 sputum samples for NGS from the 211 samples. The library

construction and sequencing process include DNA detection, library construction, library-quality detection, onboard sequencing, and quality control.

Statistical analysis

Continuous variables were expressed as means with standard deviations. The Kolmogorov-Smirnov or Shapiro-Wilk method was used to verify the non-normal distribution of the mean of two independent samples, and the Mann-Whitney U or t-test was used to perform the comparison. The chi-squared test or Fisher's exact test was used to compare the rates of qualitative data in two independent samples. The 95% confidence intervals (95%CI) were calculated using the ratio. All statistical analyses were performed with SPSS Statistics (version 23; IBM, Armonk, NY) and *P* values < 0.05 were considered statistically significant.

We calculated the screening measurements using true-positive (TP), true-negative (TN), false-positive (FP), and false-negative (FN) rates when compared to the traditional culture. Sensitivity (SE) was calculated as SE=[100%×TP/(TP+FN)], specificity (SP) as SP=[100%×TN/(TN+FP)], positive predictive value (PPV) as PPV=[100% × TP/(TP + FP)], and negative predictive value (NPV) as NPV=[100% × TN/(TN + FN)].

Results

Technical performance of the MQ-PCR assay

Analytical specificity and sensitivity

All four assays of the MQ-PCR were negative for the 24 control isolates other than the target pathogens, and there was no cross-reaction among the four assays, showing good specificity. Analyzing sensitivity at five different concentrations (5×10^3, 2.5×10^3, 1000, 500, and 100 copies/mL), when the concentration of the target pathogen is 1000 copies/mL, the detection rate of the pathogen is equal to or greater than 95%, so the

LOD is 1000 copies/mL.

Assessment of the MQ-PCR efficiency and reproducibility

According to the standard curve, the amplification efficiency of each detection index is between 95% and 100%. For the precision, the number of positive assays for the three engineered bacteria were 100% with a CV% less than 5%, and the negative had no amplification.

Verification of the MQ-PCR assay using clinical respiratory tract specimens

Patient characteristics

The demographic and clinical characteristics of the patients are summarized in Table 2. A total of 211 adult patients with LRTI were enrolled in the study cohort; 65.4% were male and the median age was 63 years (range, 18-95 years). Hospital admission was required for 73.5% (155/211), and intensive care unit (ICU) admission was required for 18.7% (29/155). The in-hospital mortality rate was 7.1% (11/155). Out of the 211 enrolled patients, 209 patients had new infiltration on chest computed tomography. The main symptoms included cough and purulent secretions (194/211; 91.9%), fever (100/211; 47.4%), dyspnea (66/211; 31.3%), hemoptysis (18/211; 8.5%), and chest pain (6/211; 2.8%). Of 211 patients who met the initial inclusion criteria, 125 (59.2%) cases had been given antimicrobial treatment. LRTI included CAP (n=117; 55.5%), HAP (n=20; 9.48%), AECOPD (n=43; 20.4%), and bronchiectasis with infection (n=31; 14.7%). Detailed clinical information about enrolled patients with LRTI is listed in Supplementary Table 2.

Pathogen detection by the traditional culture of LRT specimens

Traditional bacterial cultures identified 70 common respiratory pathogens in 63 (29.9%) specimens: *P. aeruginosa* (n=21), *K. pneumoniae* (n=13), *A. baumannii* (n=9), *H. influenzae* (n=7), *S. pneumoniae* (n=4), *S. aureus* (n=3), *E. coli* (n=3), *M. catarrhalis* (n=2), and other bacterial pathogens (n=8). Two bacterial species were identified in seven (3.3%) specimens. Detailed pathogen identification results of 211 respiratory tract specimens collected from patients with LRTI are listed in Supplementary Table 3.

Pathogen detection by the MQ-PCR assay of LRT specimens

Based on previous study (Abdeldaim et al., 2008, Kais et al., 2006), when cutoffs of $\geq 10^5$ copies/mL for sputum and $\geq 10^4$ copies/mL for BALF were applied, bacteria were detected in specimens from 136/211 (64.5%) patients (Supplementary Table 4). A total of 192 pathogens were detected and the details are summarized in Table 3. *S. pneumoniae* was most commonly detected (59/192; 30.7%)) followed by *P. aeruginosa* (37/192; 19.3%), *H. influenzae* (20/192; 10.4%), *A. baumannii* (15/192; 7.8%), *S. aureus* (13/192; 6.8%), *M. catarrhalis* (10/192; 5.2%), *K. pneumoniae* (10/192; 5.2%), and other bacterial pathogens (4/192; 2.1%). In addition, *E. faecalis* (13/192; 6.8%) and *E. faecium* (5.7%, 11/192) were also detected by MQ-PCR (Supplementary Table 5). Among the 21 patients with documented *Enterococcus* findings, 6 presented infections with *Enterococcus* alone. Of the 130 patients infected with recognized bacterial pathogens, 15 (11.5%) presented mixed infections with *Enterococcus*. A single agent was detected in 91 specimens. More than one bacterial species were identified by MQ-PCR occurred in 21.3% (45/211) specimens. Two agents were detected in 35 specimens and three agents were detected in nine specimens. Four agents were

identified in a single sputum specimen. *S. pneumoniae* was present in 59 of 211 (28%) cases, as the sole bacterial pathogen in 43 and together with other bacterial pathogens in 16 cases (Table 3).

Detection of bacterial pathogens by NGS and the MQ-PCR

The results of the twelve sputum specimens tested by NGS and MQ-PCR were consistent (Table 4). The clean data of NGS results have been uploaded to the NCBI database (accession no. **PRJNA788217**, and the sample number is consistent with Supplementary Table 3).

Streptococcus pneumoniae urine antigen test

Urine samples were collected from 72 cases in the non-antibiotic group, all of which were negative in *S. pneumoniae* urinary antigen tests.

Comparison of the MQ-PCR and traditional culture method for bacterial detection

Of the 70 pathogens in culture-positive specimens, 48 were also MQ-PCR-positive for the same species of bacteria. For the 22 organisms that MQ-PCR failed to detect, 8 were not included in our MQ-PCR assays: *Acinetobacter nosocomialis* (n=2), *Corynebacterium striatum* (n=2), *Acinetobacter johnsonii* (n=1), *Proteus mirabilis* (n=1), *Enterobacter aerogenes* (n=1), *Stenotrophomonas maltophilia* (n=1). In addition, 14 pathogens (eight *K. pneumoniae*; three *H. influenzae*; two *E.coli*; one *A. baumannii*) were culture-positive but negative by MQ-PCR. Importantly, 103 bacterial species were identified by MQ-PCR in 148 culture-negative specimens. The most frequently identified bacteria were *S. pneumoniae* (n=42), *P. aeruginosa* (n=13), and *H. influenzae*

(n=12), as shown in Table 5.

Comparison of bacteria detected by conventional culture and MQ-PCR in antibiotic use group and no antibiotic use group

Of the 125 patients who had received prior antimicrobials, 63.2% (n=79) had a bacterial pathogen detected by the MQ-PCR, but only 29.6% (n=37) were culture-positive (P<0.01). Of the 86 patients who had not received prior antimicrobials, 66.3% (n=57) had a bacterial pathogen detected by MQ-PCR, but only 30.2% (n=26) were culture-positive (P<0.01). Among patients (n=125) with antibiotic use, the most common species in the traditional culture method were *P. aeruginosa* (n=17) and *A. baumannii* (n=9), while those in MQ-PCR were also *P. aeruginosa* (n=32) and *A. baumannii* (n=15). Among those (n=86) without antibiotic use, the most common species detected by traditional culture were *K. pneumoniae* (n=8) and *H. influenzae* (n=6), but the most common bacteria in MQ-PCR were *S. pneumoniae* (n=47) and *M. catarrhalis* (n=9), as shown in Table 6.

Performance of the MQ-PCR for the identification of bacteria

The overall sensitivity, specificity, PPV, and NPV of MQ-PCR for the detection of bacteria were 77% (95%CI, 67-88%), 94% (95%CI, 93-95%), 25% (95%CI, 19-31%), and 99% (95%CI, 99-100%), respectively (Table 7). The sensitivity among the bacteria varied widely, from 100% in *S. aureus* to 0% in *E. cloacae*. Relative to sensitivity, the specificity of individual bacteria fluctuated less, ranging from 100% in *E. coli* to 73% in *S. pneumoniae*.

Discussion

In this study, we reported the development and validation of a MQ-PCR assay for twelve respiratory bacteria. The process from nucleic acid extraction to complete MQ-PCR detection takes about 3 h. The main results were as follows. Firstly, the MQ-PCR assay is more sensitive and specific than traditional culture. Furthermore, the MQ-PCR assay is time-saving, easy to use, and doesnot negatively impact by antibiotic administration before sampling. Secondly, the MQ-PCR assay improved diagnostic yield, particularly in culture-negative specimens. Thirdly, MQ-PCR can detect more co-pathogens than the traditional culture, and simultaneously provides information about individual bacterial loads. Fourthly, *S. pneumoniae* (28%, 59/211) was the most commonly identified species, especially in antibiotic-naive patients (54.7%, 47/86) at first presentation.

Polymerase chain reaction (PCR) is a simple and rapid method of identifying bacterial and viral pathogens in clinical specimens. The turnaround is typically on the scale of just a few hours (Gadsby et al., 2015). Although commercially available PCR technology currently enables a respiratory specimen to be rapidly screened for a wide range of viral (Munigala et al., 2018, Mustafa Hellou et al., 2021) and atypical bacterial pathogens (McGovern et al., 2021, Oosterheert et al., 2005, Shengchen et al., 2019), most parts of China continue to rely on conventional microbial techniques (such as Gram stain, culture, and urine antigen detection) to identify bacteria. Given that most municipal hospital laboratories in China have access to conventional real-time PCR instrumentation, we have designed this assay using multiplexed fluorescent PCR to perform specific detection of bacterial DNA in the same reaction tube. In our study, the MQ-PCR could detect 12 bacteria within 3 h with four-tube reagents, making this a cost-effective procedure.

In our study, the sensitivity, specificity, and NPV of MQ-PCR for respiratory samples

were similar to other PCR reactions, but the PPV was lower than other studies (Peiffer-Smadja et al., 2020, Roisin et al., 2018). The reason why the PPV is lower may be the low sensitivity of the traditional culture. The low diagnostic rate and low sensitivity of traditional culture are related to whether antibiotics have been used before sampling, the time from specimen collection to specimen processing. Such as for fastidious bacteria: *S. pneumoniae* and *H. influenzae*, the samples were exposed to antibiotics before sampling and processed after sample collection > 4 h, which are prone to a false negative (Ewig et al., 2002). In our study, 125 samples had been exposed to antibiotics before sampling, of which 79 samples had been used antibiotics for more than three days (Table2). In addition, the time from sample collection to delivery to the laboratory is unknown, so we can not guarantee the samples collected from the clinical microbiology laboratory to be processed within 4 h. The above may be the main reasons for the low sensitivity of traditional culture in this study, resulting in a low positive predictive value of the MQ-PCR.

Due to the well-known limitations of the traditional culture with respect to sensitivity and specificity, traditional culture may not represent a reliable reference to evaluate the performance of MQ-PCR, despite being the current gold standard. However, the results of the MQ-PCR assay in all 12 cases were supported by NGS. Therefore, we consider the results of the MQ-PCR to be reliable.

Quantification of the bacterial DNA load may be important in distinguishing infection from oropharyngeal contamination in sputum. Most studies on the quantification of bacterial DNA load to date have focused on *S. pneumoniae*, and a cutoff of 10^4–10^5

copies/mL is typically described as a significant threshold (Albrich et al., 2014, Strålin et al., 2014). A key strength of our study was the availability of other LRTI (HAP, AECOPD, and bronchiectasis with infection)-related pathogens. In this study, at cutoffs of $\geq 10^5$ copies/mL for sputum and $\geq 10^4$ copies/mL for BALF, we detected a likely bacterial etiology in 64.5% of cases, which is double the yield by conventional microbiology. This result is similar to a previous report by Gadsby et al. (Gadsby et al., 2016) in which quantitative molecular testing was used to determine the cause of pneumonia in patients and bacterial etiology was detected in 71.5% of patients. In addition, among the 22 culture-positive organisms that MQ-PCR failed to detect, 8 pathogens (*K. pneumoniae* n=7; *H. influenzae* n=1) were culture-positive, while the MQ-PCR assay was negative due to the bacterial load of fewer than 10^5 copies/mL (Table 5). This new MQ-PCR assay explores the quantitative detection of other causative agents that cause HAP, AECOPD, and bronchiectasis with infection, and lays the foundation for further research.

The new MQ-PCR assay can detect more co-pathogens than the conventional culture method. Recent studies using molecular techniques have recognized multiple bacterial pathogens in high-quality sputum samples (Gadsby et al., 2016, Wolff et al., 2017). As most molecular work done to date in the LRTI setting has focused on CAP patients, mixed-infection pathogen detection for typical bacteria may not be directly comparable to results from other recent studies in hospitalized adults with CAP (Gadsby et al., 2015, Johansson et al., 2010, Werno et al., 2012). Our total mixed-infection rate (21.3%) in the LRTI setting is lower than that of Gadsby et al. (Gadsby et al., 2016). They found

that more than one bacterial species were present in 102 (31.6%) CAP patients. Furthermore, we observed that bacterial-bacterial co-infections were more common in the hospital setting, especially in HAP patients, a finding we consider important in the proper management of LTRI. This may prove to be particularly useful in the ICU setting, where patients often are immunosuppressed or mechanically ventilated and are more likely to have nosocomial infections. But this finding needs further confirmation. Musher et al. (Musher et al., 2020) used quantitative microbiology and attention to the role of normal respiratory flora (bacteria that normally colonize the upper airways), found that normal respiratory flora appear to play a causative role in 25.8% of cases of CAP. *Enterococcus spp.* generally colonize the urethra and gastrointestinal and respiratory tracts (Savini et al., 2012). In general, *Enterococcus* spp. are not targeted by culture-based or molecular methods in the LRTI setting. However, they might be responsible for some proportion of cases of LRTI. LRTIs caused by *Enterococcus spp.* are usually nosocomial infections and often occur in immunosuppressed or mechanically ventilated patients (Huang et al., 2020, Vanschooneveld et al., 2009). In this study, we tried to expand our quantitative techniques to detect *Enterococcus* spp. We added *E. faecium* and *E. faecalis* as the target pathogens detected by the MQ-PCR, and identified 11 with *E. faecium* and 13 with *E. faecalis* in 21 patients who could provide a high-quality respiratory tract specimen. Our data are similar to those from a recent study by Musher et al. (Musher et al., 2020). They found that 13 of 68 (19.1%) patients infected with recognized bacterial pathogens were coinfected with normal respiratory flora (*Streptococcus* et al). Our work may enhance the understanding of the

etiology of LRTI, especially in patients hospitalized for LRTI. Further systematically research is needed to assess the potential role of *Enterococcus* in causing LRTI, both as the sole pathogen as well as in association with other bacteria.

In summary, the MQ-PCR assay approximately doubles bacterial pathogen detection in patients with LRTI from 29.9% to 64.5%, can detect more bacterial co-infections than the traditional culture method, and provides a simultaneous quantitative output. The new MQ-PCR assay is more sensitive than conventional identification methods and brings us closer to the mainstream adoption of quantitative molecular detection of bacteria.

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Declarations

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Ethics approval This study was approved by the Ethics Committee of the China-Japan Friendship Hospital (2019-170-K116-2).

Authors' contributions Bin Cao and Yingmei Liu contributed to the study

conception and design. Material preparation, data collection and analysis were performed by Xianxia Zhuo, Jiankang Zhao, Chunlei Wang, Binbin Li and Yanyan Fan. Lei Wang, Bin Sun and Lanhua Sun participated in the research and development of reagents. The first draft of the manuscript was written by Xianxia Zhuo and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Conflict of interest statement

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Table 1 Oligonucleotide sequences for MQ-PCR to detect target bacteria

Pathogen	Target gene	Oligonucleotide sequences	FRC* (μM)	Reference
Streptococcus pneumoniae	*lytA*	Forward: TGTTCCTGAGCAATCATCTAT Reverse: CTTGTTTCCAGYCTGTTGTT Probe: FAM-CTGGTTCTACTGCTACATCYGTTCCTTG	Forward:0.4 Forward:0.4 Probe:0.2	Ergin et al., 2009
Haemophilus influenzae	*fucK*	Forward: ACTCAACGCTTAACTGGTC Reverse: GYTAGTAAGGTTTGTCATCAT Probe: VIC-CCCGCCATTGTGTGATCTGTWGTG	Forward:0.8 Forward:0.8 Probe:0.3	Nørskov-Lauritsen, 2009
Moraxella catarrhalis	*copB*	Forward: AACCAAATYAATGACTTTACC Reverse: TTCCAACCTTTTTACCRTCCA Probe: CY5-AGGYGTGCGTGTTGACCGTT	Forward:0.4 Forward:0.4 Probe:0.25	Greiner et al., 2003
Pseudomonas aeruginosa	*gyrB*	Forward: GTGATCGCCACCCTCAAG Reverse: CGTTAGCCAGGTCGTCCA Probe: FAM-CCTGTCGCGCCTGTACCCCC	Forward:0.3 Forward:0.3 Probe:0.1	Gadsby et al., 2015
Acinetobacter baumannii	*blaOXA-51*	Forward: AGTTAAGGGAGAAYGCTACAAT Reverse: GTGGTTGGGGATGGGAT Probe: VIC-CTTGAGGCTGAACAACCCATCCAG	Forward:0.5 Forward:0.5 Probe:0.3	Nomanpour et al., 2011
Klebsiella pneumoniae	*phoE*	Forward: CTTTGTGGCTTCAACAGCGA Reverse: GTGCATGGCTTTGATCTTGC Probe: CY5-ACACATCCAGCTTGTTCGCGTTC	Forward:0.4 Forward:0.4 Probe:0.2	Fevre et al., 2011
Enterobacter cloacae	*DnaJ*	Forward: CTGCGGAAGAGCGTGAAATC Reverse: GCTTCAGCCTCTTTGTCACC Probe: CY5-AAGCGCCTGGCCATGAAATTCCAC	Forward:0.3 Forward:0.3 Probe:0.15	Chen et al., 2017
Escherichia coli	*ydiJ*	Forward: GAATCCTTGTGGGCAAATTGG Reverse: CGTGATCAGCGGTGACTATGA Probe: VIC-CCGTACAACGGGCGCTGGA	Forward:0.3 Forward:0.3 Probe:0.15	Cao et al., 2019
Burkholderia cepacia	*hisA*	Forward: CCGGCAAGCCGAAGAATC Reverse: ATCGTCTCGAGGCTGC Probe: FAM-CATTTCATCGCCGACTTCGTCGAGG	Forward:0.3 Forward:0.3 Probe:0.15	Devanga Ragupathi and Veeraraghavan, 2019
Staphylococcus aureus	*nuc*	Forward: ATTAAAGCGATTGATGGTGATA Reverse: CACTTGCTTCAGGACCATA Probe: FAM-CTCTACACCTTTTTTAGGATGCTTTGTTTCAG	Forward:0.4 Forward:0.4 Probe:0.2	Montazeri et al., 2015
Enterococcus faecium	*ddl*	Forward: GAAGTCGTAAAAGACGTAGCA Reverse: CCTAACATCGTGTAAGCTAACTT Probe: ATCGAAATGCAGATTCCAGCCGAAGT	Forward:0.4 Forward:0.4 Probe:0.3	Ozawa et al., 2000
Enterococcus faecalis	*ddl*	Forward: TGTTAGATGGAAGTGGCT Reverse: GCATGGTGTTCAATTCATT Probe: CY5-AGTCGCTGTGATTTCTTCTTAACAA	Forward:0.5 Forward:0.5 Probe:0.3	Ozawa et al., 2000

	GCA

*FRC: Final Reaction Concentration; MQ-PCR: Multiplex Quantitative PCR.

Table 2 Clinical characteristics of included patients with LRTI (n = 211)

Characteristics	N (%)
Demographics	
Male (%)	138 (65.4%)
Age, median (range) years	63 (18-95)
Clinical manifestations	
Fever	100 (47.4%)
Cough and expectoration	194 (91.9%)
Chest pain	6 (2.8%)
Dyspnea	66 (31.3%)
Hemoptysis	18 (8.5%)
Radiologic findings	209 (99.1%)
Laboratory findings	
White blood cell count [a], $\times 10^9$/L	9.16
C-reactive protein [b], mg/L (interquartile range)	71.59(7.12-124.77)
Procalcitonin [c] , ng/mL	1.45
Antimicrobial administration	
Do not receive antibiotic in the 7 day prior to sputum or BALF sampling	86(40.8%)
Received antibiotic in the 3 day prior to sputum or BALF sampling	46(21.8)
Received antibiotic ≥ 3 day prior to sputum or BALF sampling	79(37.4)
Outcome	
Intensive care unit admission [d]	29 (18.7%)
Intubation and ventilation [d]	15 (9.7%)
In-hospital mortality [d]	11 (7.1%)

[a] Information available for 204 patients. [b] Information available for 175 patients. [c] Information available for 117 patients. [d] Information available for 155 patients. LRTI: Lower respiratory tract infection. BALF: Bronchoalveolar lavage fluid.

Table 3 Pathogen detection in patients with lower respiratory tract infection using MQ-PCR method

Organisms (n=192): with ≥10^5 copies/ml for sputum with ≥10^4 copies/ml for BALF	**N (%)**	**Received antibiotic**	**Not received antibiotic**	***P value***
Streptococcus pneumoniae (sole)	43 (22.4)	9	34	0.000
S. pneumoniae + H. influenzae	5 (2.6)	3	2	1.000
S. pneumoniae + M. catarrhalis	4 (2.1)	0	4	0.026
S. pneumoniae + K. pneumoniae	1 (0.5)	0	1	0.408
S. pneumoniae + P. aeruginosa	3 (1.6)	0	3	0.066
S. pneumoniae + H. influenza + S. aureus	2 (1.0)	0	2	0.165
S. pneumoniae + H. influenza + P. aeruginosa	1 (0.5)	1	0	1.000
Haemophilus influenza (sole)	7 (3.7)	5	2	0.703
H. influenza + P. aeruginosa	2 (1.0)	1	1	1.000
H. influenza + A. baumannii	1 (0.5)	1	0	1.000

H. influenza + P. aeruginosa + S. aureus	1 (0.5)	1	0	1.000
H. influenza + A. baumannii + S. aureus	1 (0.5)	1	0	1.000
Moraxella catarrhalis (sole)	6 (3.1)	1	5	0.042
Staphylococcus aureus (sole)	3 (1.6)	2	1	1.000
S. aureus+ E. faecalis	2 (1.0)	2	0	0.515
S. aureus+ E. faecium+ E. faecalis	1 (0.5)	1	0	1.000
S. aureus+ A. baumannii	1 (0.5)	1	0	1.000
Pseudomonas aeruginosa (sole)	20 (10.4)	20	0	0.000
P. aeruginosa + E. coli	1 (0.5)	0	1	0.408
P. aeruginosa + A. baumannii	4 (2.1)	4	0	0.147
P. aeruginosa + S. aureus	1 (0.5)	1	0	1.000
P. aeruginosa + E. faecium	1 (0.5)	1	0	1.000
P. aeruginosa + E. faecalis	1 (0.5)	1	0	1.000
P. aeruginosa + A. baumannii+ E. faecalis	1 (0.5)	1	0	1.000
P. aeruginosa + K. pneumoniae + E. cloacae + E. faecalis	1 (0.5)	1	0	1.000
Acinetobacter baumannii (sole)	2 (1.0)	2	0	0.515
A. baumannii + E. faecium	2 (1.0)	2	0	0.515
A. baumannii + K. pneumoniae	1 (0.5)	1	0	1.000
A. baumannii + K. pneumoniae+ S. aureus	1 (0.5)	1	0	1.000
A. baumannii + K. pneumoniae+ E. faecium	1 (0.5)	1	0	1.000
Klebsiella pneumoniae (sole)	3 (1.6)	3	0	0.272
K. pneumoniae + E. faecium	2 (1.0)	2	0	0.515

Burkholderia cepacia (sole)	1 (0.5)	1	0	1.000
E. faecium (sole)	1 (0.5)	1	0	1.000
E. faecalis (sole)	5 (2.6)	5	0	0.081
E. faecalis + E. faecium	2 (1.0)	2	0	0.515
E. faecium + E. coli	1 (0.5)	1	0	1.000

MQ-PCR: Multiplex Quantitative PCR; BALF: Bronchoalveolar lavage fluid; *K. pneumoniae*: *Klebsiella pneumoniae*; *E. faecium*: *Enterococcus faecium*; *S. pneumoniae*: *Streptococcus pneumoniae*; *A. baumannii*: *Acinetobacter baumannii*; *H. influenzae*: *Haemophilus influenzae*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *E. cloacae*: *Enterobacter cloacae*; *E. faecalis*: *Enterococcus faecalis*; *E. coli*: *Escherichia coli*; *S. aureus*: *Staphylococcus aureus*; *B. cepacian*: *Burkholderia cepacia*; *M. catarrhalis*: *Moraxella catarrhalis*.

P value was calculated using the Chi-square test or Fisher's exact test.

Table 4 Comparison of MQ-PCR and NGS for detection of pathogens

Sample number	MQ-PCR	NGS	Kurtosis sort
94	*S. pneumoniae*	*S. pneumoniae*	26
96	*S. pneumoniae*	*S. pneumoniae*	26
99	*S. pneumoniae*	*S. pneumoniae*	23
100	*S. pneumoniae*	*S. pneumoniae*	26
103	*S. pneumoniae*	*S. pneumoniae*	24
104	*S. pneumoniae*	*S. pneumoniae*	23
106	*S. pneumoniae*	*S. pneumoniae*	26
110	*S. pneumoniae*	*S. pneumoniae*	24
111	*S. pneumoniae*	*S. pneumoniae*	21
112	*S. pneumoniae* and *P. aeruginosa*	*S. pneumoniae* and *P. aeruginosa*	23 and 30
113	*S. pneumoniae*	*S. pneumoniae*	25
150	*S. pneumoniae*	*S. pneumoniae*	26

MQ-PCR: Multiplex Quantitative PCR; NGS: Next Generation Sequencing. The sample number is consistent with the supplementary table 3. *S. pneumoniae*: *Streptococcus pneumoniae*; *P.*

aeruginosa: *Pseudomonas aeruginosa.*

Table 5 Comparison of MQ-PCR and conventional culture methods for bacterial detection

Organism isolated by conventional culture (n)	Organism detected by MQ-PCR (n)
Bacteria detected by culture but not detected by MQ-PCR (n=14)	
K. pneumoniae (5)	*K. pneumoniae* $<10^5$ copies/ml and *S. pneumoniae* (5)
K. pneumoniae (2)	*K. pneumoniae* $<10^5$ copies/ml (2)
K. pneumoniae (1)	*M. catarrhalis* (1)
H. influenzae (1)	*H. influenzae* $<10^5$ copies/ml (1)
H. influenzae (1)	*S. pneumoniae* (1)
H. influenzae (1)	No bacteria detected (1)
E. coli (1)	*A. baumannii* and *E. faecium* (1)
E. coli (1)	*H. influenzae* and *A. baumannii* (1)
A. baumannii (1)	No bacteria detected (1)
Bacterias detected by culture but the pathogens not included in the MQ-PCR assays (n=8)	
A. nosocomialis (1)	*P. aeruginosa* and *A. baumannii* (1)
A. nosocomialis (1)	*K. pneumoniae* $<10^5$ copies/ml (1)
Corynebacterium striatum (2)	No bacteria detected (2)
P. mirabilis (1)	*S. pneumoniae* and *H. influenzae* (1)
E. aerogenes (1)	*E. faecalis* (1)

A. johnsonii (1)	*E. faecium* (1)
S. maltophilia (1)	*P. aeruginosa* (1)
Specimens were culture negative, but positive for MQ-PCR (with ≥10^5 copies/ml for sputum and ≥10^4 copies/ml for BALF, n=103)	
	S. pneumoniae (n=42)
	P. aeruginosa (n=13)
	H. influenzae (n=12)
	M. catarrhalis (n=7)
	S. aureus (n=6)
Negative (148)	*K. pneumoniae* (n=4)
	A. baumannii (n=2)
	E. coli (n=1)
	B. cepacia (n=1)
	E. faecalis (n=8)
	E. faecium (n=7)

MQ-PCR: Multiplex Quantitative PCR. BALF: Bronchoalveolar lavage fluid. *K. pneumoniae*: *Klebsiella pneumoniae*; *S. pneumoniae*: *Streptococcus pneumoniae*; *M. catarrhalis*: *Moraxella catarrhalis*; *H. influenzae*: *Haemophilus influenzae*; *E. coli*: *Escherichia coli*; *A. baumannii*: *Acinetobacter baumannii*; *A. nosocomialis*: *Acinetobacter nosocomialis*; *A. johnsonii*: *Acinetobacter johnsonii*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *S. aureus*: *Staphylococcus aureus*; *E. cloacae*: *Enterobacter cloacae*; *B. cepacia*: *Burkholderia cepacia*; *E. aerogenes*: *Enterobacter aerogenes*; *S. maltophilia*: *Stenotrophomonas maltophilia*; *C. striatum*: *Corynebacterium striatum*; *P. mirabilis*: *Proteus mirabilis*; *E. faecalis*: *Enterococcus faecalis*; *E. faecium*: *Enterococcus faecium*.

Table 6 The difference of the bacteria count detected by conventional culture and MQ-PCR in antibiotic use group and no antibiotic use group

Organisms	Received antibiotic n=125		Not received antibiotic n=86	
	Conventional culture	MQ- PCR	Conventional culture	MQ- PCR
Streptococcus pneumoniae	0	12	4	47
Haemophilus influenza	1	12	6	8
Moraxella catarrhalis	0	1	2	9
Pseudomonas aeruginosa	17	32	4	5
Acinetobacter baumannii	9	15	0	0
Klebsiella pneumoniae	5	9	8	1
Enterobacter cloacae	0	1	0	0
Escherichia coli	2	1	1	1

Burkholderia cepacia	0	1	0	0
Staphylococcus aureus	2	10	1	3
Enterococcus faecium	0	11	0	0
Enterococcus faecalis	0	13	0	0
Total	36	118	26	74

MQ-PCR: Multiplex Quantitative PCR.

Table 7 Performance of the MQ-PCR for the identification of bacteria

Organisms	TP CC+=MQ-PCR+	FP CC-=MQ-PCR+	FN CC+=MQ-PCR-	TN CC-=MQ-PCR-	Sensitivity (%) [95%CI]	Sp
S. aureus	3	10	0	198	100	
S. pneumoniae	4	55	0	152	100	
E. faecium	0	11	0	200	0	
E. faecalis	0	13	0	198	0	
E. coli	1	1	2	207	33	
H. influenzae	4	16	3	188	57	
M. catarrhalis	2	8	0	201	100	
P. aeruginosa	21	16	0	174	100	
A. baumannii	8	7	1	195	89	
K. pneumoniae	5	5	8	193	39	
E. cloacae	0	1	0	210	0	
B. cepacia	0	1	0	210	0	

Total	48	144	14	2326	77% (95%CI,67-88%)	(95

TP: True positive; CC: Conventional culture; MQ-PCR: Multiplex Quantitative PCR; FP: False positive; FN: False negative; PPV: Positive predictive value; NPV: Negative predictive value; CI: confidence interval; *K. pneumoniae*: *Klebsiella pneumoniae*; *E. faecium*: *Enterococcus faecium*; *S. pneumoniae*: *Streptococcus pneumoniae*; *A. baumannii*: *Acinetobacter baumannii*; *H. influenzae*: *Haemophilus influenzae*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *E. cloacae*: *Enterobacter cloacae*; *E. faecalis*: *Enterococcus faecalis*; *E. coli*: *Escherichia coli*; *S. aureus*: *Staphylococcus aureus*; *B. cepacia*: *Burkholderia cepacia*; *M. catarrhalis*: *Moraxella catarrhalis*.

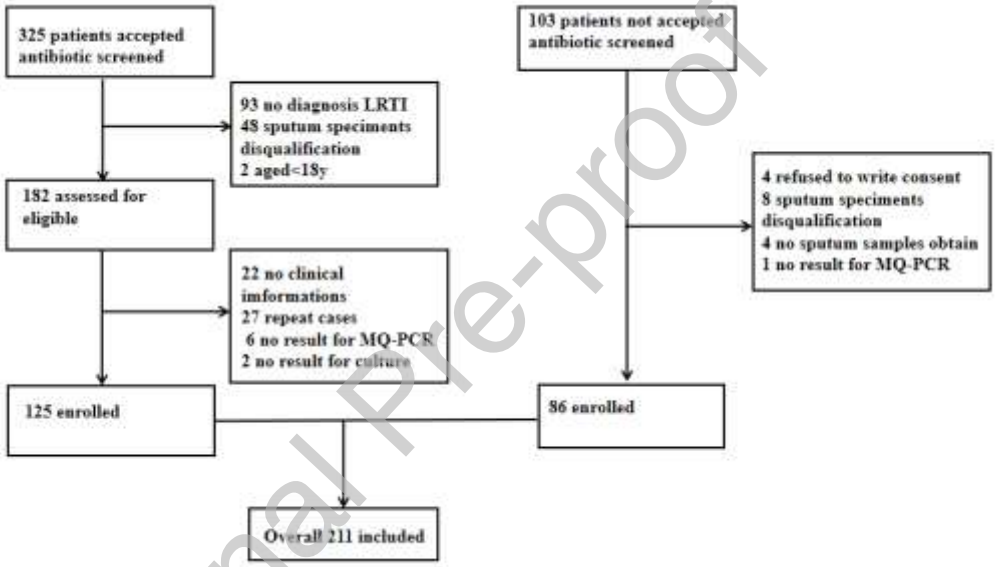

Fig. 1. Flow chart of the study.

*LRTI：lower respiratory tract infection; MQ-PCR: Multiplex Quantitative PCR.