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Development and evaluation of a multiplex quantitative polymerase chain reaction assay for detecting bacteria associated with lower respiratory tract infection

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ABSTRACT

Objectives: This study aimed to establish a multiplex quantitative polymerase chain reaction (MQ-PCR) assay for 12 bacterial pathogens found in lower respiratory tract infection (LRTI) and to evaluate its performance in a cohort 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 regarding limits of detection, reproducibility, specificity, and efficiency. In the clinical validation study, we obtained 211 sputum and/or bronchoalveolar lavage fluid (BALF) samples and detected pathogens by MQ-PCR. The MQ-PCR time was 3 h from sample collection to complete pathogen detection.

Results: The limit of detection was 1000 copies/ml, and the maximum efficiency was >95%. When cutoffs of $\geq 10^5$ copies/ml in sputum and $\geq 10^4$ copies/ml in BALF were applied, the sensitivity, specificity, and 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 mainstream adoption of quantitative molecular detection of bacteria.

Introduction

Lower respiratory tract infection (LRTI) is a substantial public health problem and a leading cause of illness and death in people of all ages worldwide (Global Burden of Diseases, Injuries, and Risk Factors (GBD) 2015 LRI Collaborators, 2017; World Health Organization, 2020). LRTIs also represent a major challenge for medical treatment because of its diverse causes. LRTIs 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 and accurate identification of pathogens is essential to guide effective treatment and prevention decisions.

The diagnosis of LRTI has greatly advanced in recent years. Many commercial multiplex polymerase chain reaction (PCR) assays for LRTI-causing viruses have been reported, such as the

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

FRC, final reaction concentration; MQ-PCR, multiplex quantitative polymerase chain reaction.

RespiFinder assay (Reijans et al., 2008) and FilmArray Respiratory Panel (Renaud et al., 2012). Cuture, although time-consuming (48-72 h) and less sensitive (Chalmers et al., 2011; Driscoll et al., 2017; England, 2014), remains the standard method for diagnosing respiratory bacteria. Although matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) can detect bacterial pathogens in about 0.5-1 h, it can identify bacteria isolated from culture plates, blood culture, or patient urine only (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 in ruling out contamination of lower respiratory tract (LRT) specimens with oral commensal flora. Most molecular work 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 mere qualitative detection of 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 sets of patients with community-acquired pneumonia (CAP) (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 the multiplex quantitative PCR (MQ-PCR) assay and evaluate its performance in LRTIs of bacterial cause through 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 traditional culture, next-generation sequencing (NGS), and the MQ-PCR assay on the same respiratory samples 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 bacterial 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.*

Primer and probe design

We used the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (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 verify optimized oligonucleotide sequences for specificity. To assess the sensitivities of species of interest, sequences were also aligned against full target-gene sequences in

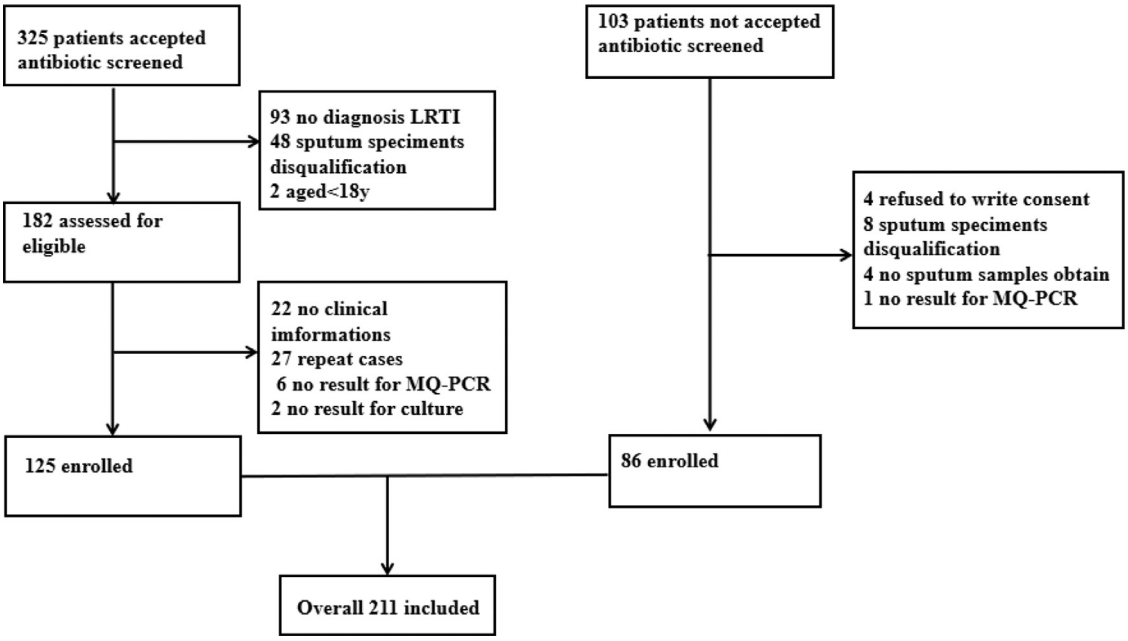

Figure 1. Flow chart of the study. LRTI, lower respiratory tract infection; MQ-PCR, multiplex quantitative polymerase chain reaction.

the GenBank database, using Primer-BLAST. Using this information, we selected 12 targets for the causative agent test and/or 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 controls 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* (Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ] 7288), *E. faecium* (ATCC BAA-472), *E. faecalis* (ATCC 33166), and *M. catarrhalis* (ATCC 25240). Plasmids including the target gene sequences were obtained from Shanghai Shenggong Biological Engineering Co., Ltd. Isometric gradient dilutions of plasmids were prepared in Tris- EDTA Buffer (Solarbio Life Sciences, Beijing, China) 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, viral, and fungal 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 concentrations of 10^6-10^9 plaque- or colony-forming units/ml. The analytical sensitivity was estimated in 20 replicates of respiratory tract specimens carrying bacteria at five different concentrations between 100 and 5000 copies/ml (Supplementary Table 1). The efficiency was measured by detecting target samples at four different concentrations (10^7, 10^6, 10^5, and 10^4 copies/ml), drawing a standard curve, and calculating amplification efficiency. The precision (reproducibility) was evaluated by comparing bacterial load quantification by the MQ-PCR assay for 10-20 replicates of 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) concentrations.

Verification of the MQ-PCR assay using clinical respiratory samples

Patient selection

Inclusion criteria for patients in the study were as follows: adult (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 use within seven days prior to sample collection (limited to outpatients); agreement to sign an informed consent form (patient or guardian); and provision of qualified sputum specimens (leukocytes >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 Chinese Thoracic Society, 2021), bronchiectasis with infection (Hill et al., 2018) and coinfection 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 a clinical microbiology laboratory. Patients with clinical and radiological evidence of LRTI were identified through electronic records review. Then the sputum or BALF samples meeting the inclusion criteria were subjected to traditional culture.

LRT specimens obtained from emergency and fever clinics. Symptomatic patients were assessed at first presentation. Patients with clinical and radiological evidence of LRTI who had not received

antibiotics within 7 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 and species were confirmed using MALDI-TOF MS (Bruker Daltonics). *S. pneumoniae* was detected in urine with antigen test. The remaining sputum or BALF from the culture was 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 by MQ-PCR.

DNA extraction

The nucleic acid extraction reagents were provided by Beijing Applied Biological Technologies Co., Ltd. (Beijing, China). We used a centrifugal column for extraction, beginning with fully liquefying the sputum (0.4% NaOH digestion solution). Then, 200 μl of clinical specimen (BALF or sputum) was processed by 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). The sample was then centrifuged for 2 s and incubated for 5 min at 37°C. After addition of 400 μl of anhydrous ethanol (Beijing Yili Fine Chemicals Co., Ltd., Beijing, China), the sample was oscillated for 15 s, centrifuged for 5 s, and incubated for 5 min at 37°C. We then centrifuged the sample for 1 min at 12,000 revolutions per minute (rpm), discarded the wastewater, perfomed two centrifugations of 30 s at 12,000 rpm with 500 μl wash (high-salt solution) and rinse (low-salt solution) buffer, and centrifuged 3 min at 12,000 rpm to remove the residual rinse 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 center of the adsorption film, incubated for 3 min at 37°C, and centrifuged at 12,000 rpm for 1 min. The nucleic acid extraction process took about 1 h.

MQ-PCR conditions

MQ-PCR reactions were performed using an ABI 7500 (Applied Biosystems, Waltham MA). The MQ-PCR assay was run in a total reaction volume of 50 µl, including 5 µl of oligonucleotide mixture (primer probes for 12 target pathogens and internal standards), 35 µl of nucleic acid amplification reaction solution (Tris hydrochloride, potassium chloride, magnesium, deoxynucleoside (uracil) triphosphate, thermostable DNA polymerase, uracil-DNA glycosylase), and 10 µl of template. The 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 and 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 55°C for 30 s. About 2 h was required to complete the MQ-PCR process. Assay runs were verified using both positive (inactivated engineered bacteria) and negative (normal saline) controls. The plasmids were diluted in equal proportions (10^4-10^7 copies/ml), and real-time PCR was performed. To quantify pathogen, plasmid concentration was plotted on the abscissa and cycle threshold (Ct) value on the ordinate to fit the standard curve. Concentrations were calculated according to the position at which the Ct 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 included 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 test 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-square test or Fisher's exact test was used to compare the rates of qualitative data in two independent samples. The 95% confidence intervals (CIs) were calculated using the confidence intervals for rates. 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 and compared with 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 MQ-PCR assays were negative for the 24 control isolates (except for the target pathogens), and there was no cross-reactivity among the four assays, demonstrating good specificity. Analysis of sensitivity at five different concentrations (5×10^3, 2.5×10^3, 1000, 500, and 100 copies/ml) shows that 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 limit of detection 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 95-100%. Regarding precision, the percentage of positive assays for the three engineered bacteria was 100%, with coefficient of variation <5%, and the negative samples showed 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 among those, intensive care unit admission was required for 18.7% (29/155). The in-hospital mortality rate was 7.1% (11/155). 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%) had been given antimicrobial treatment. LRTI included CAP (n=117; 55.5%), HAP (n=20; 9.5%), 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 traditional culture of LRT specimens

Traditional bacterial culture 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

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	
Did not receive antibiotic in the 7 days before sputum or BALF sampling	86(40.8%)46(21.8)79(37.4)
Received antibiotic in the 3 days before sputum or BALF sampling	
Received antibiotic ≥3 days before sputum or BALF sampling	
Outcome	
Intensive care unit admission[d]	29 (18.7%)
Intubation and ventilation[d]	15 (9.7%)
In-hospital mortality[d]	11 (7.1%)

BALF, bronchoalveolar lavage fluid; LRTI, lower respiratory tract infection.
[a] Information available for 204 patients. [b]Information available for 175 patients. [c]Information available for 117 patients. [d]Information available for 155 patients.

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 studies (Abdeldaim et al., 2008; Kais et al., 2006), cutoffs of $\geq 10^5$ copies/ml for sputum and $\geq 10^4$ copies/ml for BALF were applied, and bacteria were detected in specimens from 136/211 patients (64.5%) (Supplementary Table 4). In total, 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, six 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 was identified by MQ-PCR in 21.3% (45/211) of 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 59of 211 (28%) specimens, as the sole bacterial pathogen in 43 and together with other bacterial pathogens in 16 specimens (Table 3).

Detection of bacterial pathogens by NGS and MQ-PCR

The results of the 12 sputum specimens tested by NGS and MQ-PCR were consistent (Table 4). The clean NGS data have been uploaded to the NCBI database (accession no. PRJNA788217; sample number is consistent with Supplementary Table 3).

Streptococcus pneumoniae urine antigen test

Urine samples were collected from 72 patients in the no-antibiotic group. All were negative in *S. pneumoniae* urinary antigen tests.

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

Of the 70 pathogens in culture-positive specimens, 48 were also detected in the same specimens by MQ-PCR. Of the 22 organisms not detected by MQ-PCR, eight 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), and *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 and no-antibiotic use groups

Of the 125 patients who had previously received antimicrobials, 63.2% (n=79) had a bacterial pathogen detected by MQ-PCR, but only 29.6% (n=37) were culture-positive (P <0.01). Of the 86 patients who had not previously received 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 detected by the traditional culture method were *P. aeruginosa* (n=17) and *A. baumannii* (n=9); those detected by MQ-PCR were also *P. aeruginosa* (n=32) and *A. baumannii* (n=15). Among patients (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 detected by 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 the 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 for individual bacteria fluctuated less, ranging from 100% in *E. coli* to 73% in *S. pneumoniae*.

Discussion

In this study, we report the development and validation of an MQ-PCR assay for 12 respiratory bacteria. The process from nucleic acid extraction to complete MQ-PCR detection takes about 3 h. The main results are as follows. First, the MQ-PCR assay is more sensitive and specific than traditional culture. Furthermore, the MQ-PCR assay is time-saving, easy to use, and is not negatively impacted by antibiotic administration before sampling. Second, the MQ-PCR assay improves diagnostic yield, particularly in culture-negative specimens. Third, the MQ-PCR can detect more copathogens than can traditional culture, and simultaneously provides information about individual bacterial loads. Fourth, *S. pneumoniae* (28%; 59/211) was the most commonly identified species, especially in antibiotic-naive patients (54.7%; 47/86) at first presentation.

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

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

Organisms (n=192) with ≥10^5 copies/ml in sputum and ≥10^4 copies/ml in BALF	N (%)	Antibiotic received	No antibiotic received	P- value[a]
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

A. baumanni, Acinetobacter baumannii; BALF, Bronchoalveolar lavage fluid; *E. cloacae, Enterobacter cloacae; E. coli, Escherichia coli; E. faecalis, Enterococcus faecalis; E. faecium, Enterococcus faecium; H. influenzae, Haemophilus influenzae; K. pneumoniae, Klebsiella pneumoniae; M. catarrhalis, Moraxella catarrhalis*; MQ-PCR, multiplex quantitative polymerase chain reaction; *P. aeruginosa, Pseudomonas aeruginosa; S. aureus, Staphylococcus aureus; S. pneumoniae, Streptococcus pneumoniae*.
[a] *P*-value was calculated using the chi-square test or Fisher exact test.

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

Sample number[a]	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 polymerase chain reaction; NGS, next-generation sequencing; *P. Aeruginosa, Pseudomonas aeruginosa; S. Pneumoniae, Streptococcus pneumoniae*.
[a] The sample number is consistent with Supplementary Table 3.

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 regions of China continue to rely on conventional microbiological 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 detection of specific bacterial DNAs in the same reaction tube. In our study, the MQ-PCR could detect 12 bacteria within 3 h with four reagent tubes, 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 in other studies (Peiffer-Smadja et al., 2020;

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* (1)*H. influenzae* (1)	*H. influenzae* $<10^5$ copies/ml (1)*S. pneumoniae* (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)
Bacteria detected by culture but not included in MQ-PCR assays (n=8)	
A. nosocomialis (1)*A. nosocomialis* (1)	*P. aeruginosa* and *A. baumannii* (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)
Bacteria not detected by culture but detected by MQ-PCR ($\geq 10^5$ copies/ml in sputum and $\geq 10^4$ copies/ml in BALF, n=103)	
Negative (148)	*S. pneumoniae* (n=42)
	P. aeruginosa (n=13)*H. influenzae* (n=12)*M. catarrhalis* (n=7)
	S. aureus (n=6)
	K. pneumoniae (n=4)*A. baumannii* (n=2)
	E. coli (n=1)
	B. cepacia (n=1)*E. faecalis* (n=8)
	E. faecium (n=7)

A. baumannii, Acinetobacter baumannii; A. johnsonii, Acinetobacter johnsonii; A. nosocomialis, Acinetobacter nosocomialis; B. cepacia, Burkholderia cepacia; BALF, bronchoalveolar lavage fluid; *E. aerogenes, Enterobacter aerogenes; E. coli, Escherichia coli; E. faecalis, Enterococcus faecalis; E. faecium, Enterococcus faecium; H. influenzae, Haemophilus influenzae; K. pneumoniae, Klebsiella pneumoniae; M. catarrhalis, Moraxella catarrhalis*; MQ-PCR, multiplex quantitative polymerase chain reaction; *P. aeruginosa, Pseudomonas aeruginosa; P. mirabilis, Proteus mirabilis; S. aureus, Staphylococcus aureus; S. maltophilia, Stenotrophomonas maltophilia; S. pneumoniae, Streptococcus pneumoniae*.

Table 6
The difference in bacterial count detected by conventional culture and MQ-PCR in antibiotic and no-antibiotic use groups.

Organism	Received antibiotic n=125		Did not receive 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 polymerase chain reaction.

Roisin et al., 2018). The reason for lower PPV may be the low sensitivity of traditional culture. The low diagnostic rate and low sensitivity of traditional culture are related to antibiotic use before sampling and to the time from specimen collection to processing. For fastidious bacteria such as *S. pneumoniae* and *H. influenzae*, patient exposure to antibiotics before sampling and sample processing >4 h after collection contribute to false negatives (Ewig et al., 2002). In our study, 125 samples were from patients who had been exposed to antibiotics before sampling, 79 of whom had used antibiotics for more than three days (Table 2). In addition, the time from sample collection to delivery to the laboratory is unknown, so we cannot guarantee that samples collected from the clinical microbiology laboratory were processed within 4 h.

Because of the well-known limitations of traditional culture with respect to sensitivity and specificity, it may not represent a reliable reference to evaluate the performance of the MQ-PCR, despite its status as the current gold standard. However, the results of the MQ-PCR assay in all 12 bacteria 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 rate by conventional microbiology. This result is similar to a previous report by 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, eight pathogens (*K. pneumoniae* n=7; *H. influenzae*

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

Organisms	TPCC+=MQ-PCR+	FPCC-=MQ-PCR+	FNCC+=MQ-PCR-	TNCC-=MQ-PCR-	Sensitivity (%)[95% CI]	Specificity (%)[95% CI]	PPV (%)[95% CI]	NPV (%)[95% CI]
S. aureus	3	10	0	198	100	95	23	100
S. pneumoniae	4	55	0	152	100	73	7	100
E. faecium	0	11	0	200	0	95	0	100
E. faecalis	0	13	0	198	0	94	0	100
E. coli	1	1	2	207	33	100	50	99
H. influenzae	4	16	3	188	57	92	20	98
M. catarrhalis	2	8	0	201	100	96	20	100
P. aeruginosa	21	16	0	174	100	92	57	100
A. baumannii	8	7	1	195	89	97	53	100
K. pneumoniae	5	5	8	193	39	98	50	96
E. cloacae	0	1	0	210	0	100	0	100
B. cepacia	0	1	0	210	0	100	0	100
Total	48	144	14	2326	77% (95%CI,67-88%)	94% (95%CI,93-95%)	25% (95%CI,19-31%)	99% (95%CI,99-100%)

CC, conventional culture; CI, confidence interval; FN, false negative; FP, false positive; TN, true negative; MQ-PCR, multiplex quantitative polymerase chain reaction; NPV, negative predictive value; PPV, positive predictive value; TP, true positive; *A. Baumannii, Acinetobacter baumannii; B. cepacia, Burkholderia cepacia; E. cloacae, Enterobacter cloacae; E. coli, Escherichia coli; E. faecalis, Enterococcus faecalis; E. faecium, Enterococcus faecium; H. influenzae, Haemophilus influenzae; K. pneumoniae, Klebsiella pneumoniae; M. catarrhalis, Moraxella catarrhalis; P. Aeruginosa, Pseudomonas aeruginosa; S. Aureus, Staphylococcus aureus; S. Pneumoniae, Streptococcus pneumoniae.*

n=1) were MQ-PCR-negative because the bacterial load was $<10^5$ copies/ml (Table 5). This new MQ-PCR assay explores the quantitative detection of other causative agents of HAP, AECOPD, and bronchiectasis with infection, and lays the foundation for further research.

The new MQ-PCR assay can detect more copathogens 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., 2016). 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 in Gadsby et al. (2016). They found that more than one bacterial species was present in 102 CAP patients (31.6%). Furthermore, we observed that bacterial coinfections were more common in the hospital setting, especially in HAP patients; we consider this finding important in the proper management of LRTI. This may prove to be particularly useful in the intensive care unit setting, where patients often are immunosuppressed or mechanically ventilated and are more likely to have nosocomial infections. However, this finding needs further confirmation.

Musher et al. (2020), using quantitative microbiology and with 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 CAP cases. *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 may be responsible for some proportion of LRTI cases. 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 MQ-PCR, and among 21 patients who could provide a high-quality respiratory tract specimen we identified 11 with *E. faecium* and 13 with *E. faecalis*. Our data are similar to those from a recent study by Musher et al. (2020). They found that of patients infected with recognized bacterial pathogens, 19.1% (13/68) were coinfected with normal respiratory flora (*Streptococcus*, etc.). Our work may enhance the understanding of the etiology of LRTI, especially in hospitalized patients with LRTI. Further systematic 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 the bacterial pathogen detection rate in patients with LRTI from 29.9% to 64.5%, can detect more bacterial coinfections 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.

Conflicts of interest

Lei Wang, Bin Sun, and Lanhua Sun are employed by Beijing Applied Biological Technologies Co., Ltd. To our knowledge, the rest of the authors have no potential conflicts of interest.

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Ethical approval

This study was approved by the Ethics Committee of the China-Japan Friendship Hospital (2019-170-K116-2). Informed consent was signed by participating patients or their guardians.

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Authors contributions

Bin Cao and Yingmei Liu contributed to the study conception and design. Materials 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.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2022.05.052.

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