Development of two real-time multiplex PCR assays for the detection and quantification of eight key bacterial pathogens in lower respiratory tract infections

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Abstract

The frequent lack of a positive and timely microbiological diagnosis in patients with lower respiratory tract infection (LRTI) is an important obstacle to antimicrobial stewardship. Patients are typically prescribed broad-spectrum empirical antibiotics while microbiology results are awaited, but, because these are often slow, negative, or inconclusive, de-escalation to narrow-spectrum agents rarely occurs in clinical practice. The aim of this study was to develop and evaluate two multiplex real-time PCR assays for the sensitive detection and accurate quantification of *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus*, *Moraxella catarrhalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. We found that all eight bacterial targets could be reliably quantified from sputum specimens down to a concentration of 100 CFUs/reaction (8333 CFUs/mL). Furthermore, all 249 positive control isolates were correctly detected with our assay, demonstrating effectiveness on both reference strains and local clinical isolates. The specificity was 98% on a panel of nearly 100 negative control isolates. Bacterial load was quantified accurately when three bacterial targets were present in mixtures of varying concentrations, mimicking likely clinical scenarios in LRTI. Concordance with culture was 100% for culture-positive sputum specimens, and 90% for bronchoalveolar lavage fluid specimens, and additional culture-negative bacterial infections were detected and quantified. In conclusion, a quantitative molecular test for eight key bacterial causes of LRTI has the potential to provide a more sensitive decision-making tool, closer to the time-point of patient admission than current standard methods. This should facilitate de-escalation from broad-spectrum to narrow-spectrum antibiotics, substantially improving patient management and supporting efforts to curtail inappropriate antibiotic use.

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Introduction

The frequent lack of a positive and timely microbiological diagnosis in patients with lower respiratory tract (LRT) infection (LRTI) is an important obstacle to antimicrobial stewardship. Patients are typically prescribed broad-spectrum empirical antibiotics while microbiology results are awaited, but, because these are often slow, negative, or inconclusive, de-escalation to narrow-spectrum agents rarely occurs in clinical practice.

Current standard diagnostic methods for respiratory bacteria are culture-based and typically take 24–72 h [1]. Culture also has low sensitivity; a positive microbiological diagnosis may only be made in approximately 30% of patients with community-acquired pneumonia [2]. As a wide range of

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pathogens can cause LRTI, it is common to request a mixture of different diagnostic tests, including culture, antigen testing, and serology, on a number of different specimen types [3]. In contrast, the routine use of viral multiplex real-time PCR (mRT-PCR) assays allows a single respiratory specimen to be screened for a large number of respiratory viruses easily within the working day [4].

We sought to improve the sensitivity and turn-around time of microbiological diagnosis of LRTI in our centre by developing a quantitative bacterial mRT-PCR approach. At the same time, we wanted to simplify the process by testing the same LRT specimen extract used for viral mRT-PCR and using the same PCR platforms, thus fitting in with our current workflow and minimizing costs. A key requirement was the ability to determine the bacterial load in order to provide information that may be clinically useful in excluding LRT specimen contamination with oral commensal bacteria.

Real-time PCR assays for some common respiratory bacteria have been described, but no single assay covers the wide range of Gram-positive and Gram-negative pathogens required for LRTI diagnosis. Furthermore, some assays have a lack of sensitivity and/or specificity for organisms such as *Streptococcus pneumoniae* and *Haemophilus influenzae*, owing to the use of suboptimal gene targets [3,5–10]. Several molecular respiratory pathogen panels are currently commercially available or in development. Typically, these are more expensive than PCRs developed in-house, lack a full range of viral and bacterial targets, are non-quantitative, or require specific testing platforms [11–13].

The aim of this study was to develop and evaluate two mRT-PCR assays for the sensitive detection and accurate quantification of the following eight respiratory bacterial pathogens: *S. pneumoniae*, *H. influenzae*, *Staphylococcus aureus*, and *Moraxella catarrhalis* (mRT-PCR 1), and *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* (mRT-PCR 2).

Materials and methods

Positive and negative control isolates

The positive control bacterial strains used in assay validation were as follows: *S. pneumoniae* American Type Culture Collection (ATCC) 49619, *H. influenzae* ATCC 9007, *Staphylococcus aureus* ATCC 29213, *M. catarrhalis* local laboratory reference strain, *E. coli* ATCC 35218, *K. pneumoniae* National Collection of Type Cultures (NCTC) 13442, *P. aeruginosa* ATCC 27853, and *A. baumannii* NCTC 13424. Phocine herpes virus (PhHV) was obtained as a viral cell culture stock from the Department of Virology, University Hospital Rotterdam, for use as an internal control. Plasmids containing assay target genes were generated by cloning PCR products with the pGEM-T Easy vector system (Promega, Southampton, UK). Plasmid extracts were diluted in carrier polyA RNA (Qiagen, Manchester, UK) at 0.05 mg/L in ten-fold dilution series for use in PCR optimization and as quantification standards. A large panel of control isolates were selected to include organisms targeted by the mRT-PCR 1 and mRT-PCR 2 assays, and 88 isolates closely related to the target organisms and/or commonly found in the respiratory tract as pathogens or commensals (Table 1). Isolates were obtained from the Royal Infirmary of Edinburgh Clinical Microbiology Laboratory, Scottish *Haemophilus*, *Legionella*, Meningococcus and Pneumococcus Reference Laboratory, Scottish MRSA Reference Laboratory, Scottish Bacterial Sexually Transmitted Infection Reference Laboratory, ATCC and NCTC Public Health England. *Chlamydophila pneumoniae* and *Chlamydophila psittaci* were commercially supplied as DNA extracts (Vircell, Granada, Spain). Well-characterized clinical isolates were from respiratory specimens wherever possible, and were identified by colonial morphology, standard biochemical methods, VITEK-2 (bioMérieux, Basingstoke, UK), Microflex matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Bruker, Coventry, UK), and sequencing, as appropriate.

Nucleic acid extraction

Pure cultures of control bacterial isolates were suspended in saline to 0.5 McFarland standard concentration, and total nucleic acid was extracted with the DNeasy Blood and Tissue kit (Qiagen), following the protocol for Gram-positive bacteria according to the manufacturer's instructions. Crude cell lysates were also made by boiling 150 µL of cell suspension for 10 min, centrifuging for 1 min at 11 000 **g**, and removing the supernatant for testing. Sputum specimens were initially centrifuged to pellet purulent material, and then physically homogenized in sterile viral transport medium with sterile glass beads. Total nucleic acid was extracted from 200 µL of clinical specimen with the following protocol: 60 min of incubation with 4 µL of enzymatic lysis buffer (20 mM Tris, pH 8, 2 mM EDTA, 1.2% Triton X-100) + 2 µL of lysozyme (100 mg/mL) at 37°C, 60 min of incubation with 25 µL of proteinase K (Qiagen) at 56°C, nucliSENS easyMAG (bioMérieux) automated extraction with PhHV internal control added to the lysis buffer, and elution in 100 µL.

Assay design

Candidate assay targets for the eight bacteria of interest were chosen on the basis of published validation data. The National Centre for Biotechnology Information standard nucleotide Basic Local Alignment Search Tool was used to check candidate oligonucleotide sequence matches to targets in the GenBank database. Assays were then redesigned or modified with Beacon Designer (Premier Biosoft, Palo Alto, CA, USA) and

TABLE 1. Specificity panel

Organism	No.	Source/strain
Positive control isolates		
Streptococcus pneumoniae	36	Clinical isolates (25); SHLMPRL isolates (10); ATCC 49619
Haemophilus influenzae	47	Clinical isolates (25); SHLMPRL isolates (18) (serotypes A, B, D, E, F, NTHi); ATCC 9007 (serotype C); ATCC 49766 (NTHi); ATCC 49247 (NTHi); NCTC 8468
Staphyloccoccus aureus	41	Clinical isolates (25); SMRSARL isolates (10); MRSA S113; ATCC 25923; ATCC 1026; ATCC BAA-976; ATCC BAA-977; ATCC 29213
Moraxella catarrhalis	26	Clinical isolates (25); NCTC 11020
Escherichia coli	28	Clinical isolates (25); NCTC 13476; ATCC 25922; ATCC 35218
Klebsiella pneumoniae	28	Clinical isolates (25); NCTC 13442; NCTC 13443; NCTC 13439
Pseudomonas aeruginosa	26	Clinical isolates (25); ATCC 27853
Acinetobacter baumannii	17	Clinical isolates (16); NCTC 13424
Negative control isolates (n = 88)		
Abiotrophia adiacens	1	NCTC 13000
Acinetobacter calcoaceticus	1	Clinical isolate
Acinetobacter haemolyticus	1	NCTC 12155
Acinetobacter. johnsonii	1	NCTC 12154
Acinetobacter junii	1	NCTC 12153
Acinetobacter lwoffii	1	Clinical isolate
Acinetobacter nosocomialis	1	Clinical isolate
Actinomyces israelii	1	Clinical isolate
Aggregatibacter actinomycetemcomitans	1	NCTC 9710
Aggregatibacter segnis	1	SHLMPRL isolate
Arcanobacterium haemolyticum	1	Clinical isolate
Bordetella parapertussis	1	NCTC 5952
Bordetella pertussis	1	NEQAS 1505
Chlamydophila pneumoniae (DNA)	1	CM-1 (VR1360)
Chlamydophila psittaci (DNA)	1	6BC
Corynebacterium striatum	1	NCTC 764
Eikenella corrodens	1	ATCC BAA-1152
Enterobacter hormacecei	1	ATCC 700323
Enterococcus casseliflavus	1	ATCC 700327
Enterococcus faecalis	1	ATCC 51299
Fusobacerium necrophorum	1	Clinical isolate
Gemella morbillorum	1	Clinical isolate
Haemophilus aphrophilus	1	Clinical isolate
Haemophilus haemolyticus	1	NCTC 10659
Haemophilus parahaemolyticus	1	Clinical isolate
Haemophilus parainfluenzae	10	Clinical isolate
Haemophilus paraphrohaemolyticus	1	NCTC 10670
Kingella kingae	1	NCTC 10529
Klebsiella oxytoca	1	Clinical isolate
Legionella pneumophila	1	Clinical isolate
Micrococcus luteus	1	Clinical isolate
Moraxella atlantae	1	NCTC 11091
Moraxella lacunata	1	NCTC 11011
Moraxella nonliquefaciens	1	NCTC 10464
Moraxella osloensis	1	Clinical isolate
Mycoplasma pneumoniae	1	NCTC 10119
Mycoplasma salivarium	1	NCTC 10113
Neisseria cinerea	1	SBSTIRL isolate
Neisseria gonorrhoeae	1	SBSTIRL isolate
Neisseria lactamica	1	SBSTIRL isolate
Neisseria meningitidis	1	SBSTIRL isolate
Neisseria mucosa	1	SBSTIRL isolate
Neisseria perflava	1	SBSTIRL isolate
Neisseria sicca	1	SBSTIRL isolate
Neisseria subflava	1	SBSTIRL isolate
Peptostreptococcus magnus	1	Clinical isolate
Porphyromonas gingivalis	1	NCTC 11834
Prevotella intermedia	1	NCTC 13070
Pseudomonas fluorescens	1	NCTC 10038
Pseudomonas putida	1	NCTC 10936
Pseudomonas stutzeri	1	NCTC 10475
Staphylococcus capitis	1	SMRSARL isolate
Staphylococcus epidermidis	3	ATCC 12228, SMRSARL isolate (2)
Staphylococcus haemolyticus	2	SMRSARL isolate
Staphylococcus hominis	1	SMRSARL isolate
Staphylococcus intermedius	1	SMRSARL isolate
Staphylococcus lugdunensis	1	SMRSARL isolate
Staphylococcus pseudintermedius	1	SMRSARL isolate
Staphylococcus saphrophyticus	1	Clinical isolate
Staphylococcus warneri	1	SMRSARL isolate
Stenotrophomonas maltophila	1	ATCC 17666
Streptococcus agalactiae	1	Clinical isolate
Streptococcus anginosus	1	Clinical isolate
Streptococcus bovis	1	NCTC 8177
Streptococcus constellatus	1	Clinical isolate
Streptococcus gordonii	1	Clinical isolate
Streptococcus intermedius	1	Clinical isolate
Streptococcus mitis	1	SHLMPRL isolate
Streptococcus mutans	1	Clinical isolate
Streptococcus oralis	1	Clinical isolate
Streptococcus parasanguinis	2	SHLMPRL isolate, clinical isolate
Streptococcus pyogenes	1	Clinical isolate
Streptococcus salivarius	1	ATCC 19258
Streptococcus sanguinis	1	Clinical isolate
Ureaplasma urealyticum	1	NCTC 10177

ATCC, American Type Culture Collection; MRSA, methicillin-resistant *Staphylococcus aureus*; NCTC, National Collection of Type Cultures, Public Health England; NTHi, non-typeable *Haemophilus influenzae*; SBSTIRL, Scottish Bacterial Sexually Transmitted Infection Reference Laboratory; SHLMPRL, Scottish *Haemophilus*, *Legionella*, Meningococcus and Pneumococcus Reference Laboratory; SMRSARL, Scottish MRSA Reference Laboratory.

Autodimer [14] in order to optimize for multiplex performance. Optimized oligonucleotide sequences were checked by the Basic Local Alignment Search Tool against the GenBank database for *in silico* specificity. Sequences were also checked against alignments of all target gene sequences deposited in GenBank for the species of interest to check *in silico* sensitivity. On the basis of these assessments, eight targets were selected for pathogen detection, with four targets per mRT-PCR assay. The composition of each mRT-PCR assay is detailed in Table 2. Discrimination of each target in the reaction was achieved with the use of oligonucleotide probes labelled with one of four fluorophores: 6-FAM, Texas Red, Yakima Yellow, and Cy5. In order to assess the quality of LRT specimens, a real-time quantitative PCR assay was also designed with Beacon Designer (Premier Biosoft) and the RefSeq gene NG_007073.2 for the detection of the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene. An already validated in-house real-time PCR assay for the PhHV *gB* gene was used to detect PCR inhibition [15].

Real-time PCR

Reactions were carried out on the ABI 7500 Fast instrument (Applied Biosystems, Paisley, UK). mRT-PCR assays were carried out in a total volume of 20 μL, comprising 10 μL of QuantiFast Multiplex PCR mastermix (Qiagen), 2 μL of nuclease-free water (Promega), 2 μL of oligonucleotide mixture (Table 2), and 6 μL of nucleic acid extract. Cycle parameters were 95°C for 5 min, followed by 45 cycles of 95°C for 45 s and 60°C for 45 s. *GAPDH* real-time PCR was carried out with the same reaction components, but with different cycle parameters: 95°C for 5 min, followed by 45 cycles of 95°C for 30 s and 60°C for 30 s. PhHV PCR was carried out with 10 μL of Express qPCR Universal SuperMix (Invitrogen, Paisley, UK), 1 μL of oligonucleotide mixture (Table 2), and 9 μL of nucleic

TABLE 2. Oligonucleotide sequences

Assay	Organism	Gene target	Oligonucleotide	Final reaction concentration (μM)	Reference
mRT-PCR 1	*Streptococcus pneumoniae*	Autolysin (*lytA*)	Forward: ACGCAATCTAGCAGATGAAGCA	0.10	[17]
			Reverse: TCGTGCGTTTTAATTCCAGCT	0.10	
			Probe: YY-TGCCGAAAACGCTTGATACAGGGAG-BHQ1	0.05	
	Haemophilus influenzae	L-Fuculokinase (*fucK*)	Forward: ATGGCGGGAACATCAATGA	0.15	[20]
			Reverse: ACGCATAGGAGGGAAATGGTT	0.15	
			Probe: FAM-CGGTAATTGGGATCCAT-MGB	0.10	
	Staphylococcus aureus	Thermostable nuclease (*nuc*)	Forward: AGCATCCTAAAAAAGGTGTAGAGA	0.15	[22]
			Reverse: CTTCAATTTTMTTTGCATTTTCTACCA	0.15	
			Probe: TEX-TTTTCGTAAATGCACTTGCTTCAGGACCA-BHQ2	0.10	
	Moraxella catarrhalis[a]	Outer membrane protein (*copB*)	Forward: CGTGTTGACCGTTTTGACTTT	0.15	Modified from [23]
			Reverse: CATAGATTAGGTTACCGCTGACG	0.15	
			Probe: Cy5-ACCGACATCAACCCAAGCTTTGG–BHQ3a	0.10	
mRT-PCR 2	*Escherichia coli*[b]	Conserved protein, function unknown (*yccT*)	Forward: ATCGTGACCACCTTGATT	0.25	Modified from [24]
			Reverse: TACCAGAAGATCGACATC	0.25	
			Probe: TEX-CATTATGTTTGCCGGTATCCGTTT-BHQ2	0.10	
	Klebsiella pneumoniae	Citrate synthase (*gltA*)	Forward: AGGCCGAATATGACGAAT	0.25	Modified from [24]
			Reverse: GGTGATCTGCTCATGAA	0.25	Probe: this publication
			Probe: YY-ACTACCGTCACCCGCCACA-BHQ1	0.10	
	Pseudomonas aeruginosa	DNA gyrase subunit B (*gyrB*)	Forward: CCTGACCATCCGTCGCCACAAC	0.25	[25]
			Reverse: CGCAGCAGGATGCCGACGCC	0.25	
			Probe: FAM-CCGTGGTGGTAGACCTGTTCCCAGACC-BHQ1	0.10	
	Acinetobacter baumannii	OXA-51-like β-lactamase (bla_{OXA-51}-like)	Forward: TTTAGCTCGTCGTATTGGACT	0.125	Modified from [28]
			Reverse: CCTCTTGCTGAGGAGTAATTTT	0.125	
			Probe: Cy5-TGGCAATGCAGATATCGGTACCCA-BHQ3a	0.05	
Specimen quality control	Human	Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	Forward: TTGTCTCACTTGTTCTCT	0.30	This publication
			Reverse: ATGGGAGTTGTTTTCTTG	0.30	
			Probe: FAM-CTCGTCTTCTGTCATCTCTGCTG-BHQ1	0.20	
Internal control for inhibition	PhHV	Glycoprotein B (*gB*)	Forward: GGGCGAATCACAGATTGAATC	0.30	[15]
			Reverse: GCGGTTCCAAACGTACCAA	0.30	
			Probe:Cy5-TTTTTATGTGTCCGCCACCATCTGGATC–BHQ3a	0.10	

Fluorophores: BHQ, Black Hole Quencher; TEX, Texas Red; YY, Yakima Yellow.
mRT-PCR, multiplex real-time PCR; PhHV, phocine herpes virus.
[a]*Moraxella catarrhalis* assay detects *Moraxella lacunata* and *Moraxella nonliquefaciens*.
[b]*Escherichia coli* assay detects *Shigella* species.

acid extract. Cycle parameters were 95°C for 20 s, followed by 45 cycles of 95°C for 3 s and 60°C for 30 s. Runs were accepted if negative (no template) controls were negative and positive controls for each amplification target were positive. For quantification, mixed plasmid dilution series ranging from 6×10^1 to 6×10^6 gene copies/reaction were included in each run. ABI 7500 Fast System SDS software v. 1.4 (Applied Biosystems) was used to construct a six-point standard curve and extrapolate a quantitative result. Runs were accepted if standard curves were linear (reaction efficiency of 90–119%, $R^2 > 0.98$). Clinical specimens were classified as positive and quantifiable for a bacterial target if the bacterial load was ≥100 CFUs/reaction. Quantitative results were accepted if the internal control was positive with a quantification cycle (Cq) value within the range ±1 log (Cq ± 3.33) difference from negative extraction controls. CFUs/reaction were assumed to be equivalent to gene copies/reaction, because all of the bacterial target genes are single copy. Conversion of gene copies/reaction to CFUs/mL was based on a 6-μL input per PCR reaction drawn from 100 μL of nucleic acid extract concentrated from 200 μL of specimen.

Analytical performance

PCR efficiency ($E = 10^{(-1/\text{slope})} - 1$) was estimated by testing at least ten replicates of ten-fold mixed plasmid dilution series in different runs, and was acceptable between the values of 0.90 and 1.10. The quantitative range was determined from the range of ten-fold mixed plasmid dilutions that gave optimal linearity, efficiency, standard deviation and coefficient of variation between replicates. Analytical specificity was measured by testing DNA extracted from the large panel of positive and negative control isolates detailed above. Analytical sensitivity was estimated with surplus anonymized sputum specimens spiked before extraction with target bacterial species (mock specimens). These were tested at five different bacterial spike concentrations in triplicate per run, for four runs. A probit analysis was carried out to estimate the limit of detection of each assay (Minitab v.17). Precision by repeatability was used as a proxy for analytical accuracy, as reference standards for quantification of the eight bacterial pathogens are not available. To encompass between-run and within-run variation in both the extraction process and the PCR process, mock specimens at three different bacterial spike concentrations were tested in triplicate per run, for four runs on different days, with the same operator and equipment. To assess performance in quantification of mixed infections, plasmids were mixed to give unequal mixtures for three targets at 200 000, 20 000 and 200 gene copies/reaction. This was carried out for every combination of the four targets for both the mRT-PCR 1 assay and the mRT-PCR 2 assay (Table 3).

TABLE 3. Matrix for triple mixture composition

Mixture	200 gene Copies/reaction	20 000 gene copies/reaction	200 000 gene copies/reaction
1	A	B	C
2	A	B	D
3	A	C	D
4	A	D	C
5	A	C	B
6	A	D	B
7	B	A	C
8	B	A	D
9	B	C	D
10	B	D	C
11	B	C	A
12	B	D	A
13	C	A	B
14	C	A	D
15	C	B	D
16	C	D	B
17	C	B	A
18	C	D	A
19	D	A	B
20	D	A	C
21	D	B	C
22	D	C	B
23	D	B	A
24	D	C	A

For multiplex real-time PCR (mRT-PCR) 1: A = *Streptococcus pneumoniae*; B = *Haemophilus influenzae*; C = *Staphylococcus aureus*; D = *Moraxella catarrhalis*.
For mRT-PCR 2: A = *Escherichia coli*; B = *Klebsiella pneumoniae*; C = *Pseudomonas aeruginosa*; D = *Acinetobacter baumannii*.

Performance on clinical LRT specimens

Anonymized residual sputum specimens from patients with radiologically confirmed pneumonia and bronchalveolar lavage (BAL) fluid specimens from critical-care patients were collected between September 2012 and March 2015 following routine microbiological culture. For sputum specimens, a fleck of pus was inoculated directly onto Columbia horse blood agar and chocolate blood bacitracin agar (Oxoid, Basingstoke, UK), spread for the production of discrete colonies, and incubated for 48 h at 37°C in 5% CO_2; microscopy was not carried out. Isolates were identified with standard biochemical methods and/or matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Results for sputum specimens were reported semiquantitatively in the following format: small numbers—colonies present in the well of the plate only; moderate numbers—colonies present in the first streak out from the well; and large numbers—colonies present in the second or third streak out from the well. Microbiological culture of BAL fluid specimens was carried out as for sputum specimens, with the exception that cultures were quantitative; plates were inoculated evenly with 10 µL of fluid, and results were reported as CFUs/mL. Residual specimens were stored at −80°C before being tested retrospectively for this study. Testing was carried out in accordance with local ethical approval (South East Scotland SAHSC Human Annotated Bio-Resource reference No. 10/S1402/33). Datasets were subjected to a normality test, and differences between non-parametric data were tested for significance with the Mann–Whitney test (Minitab v.17).

Results

Assay design

The eight bacterial pathogens chosen for the mRT-PCR 1 and mRT-PCR 2 assays were the species most commonly detected by conventional culture-based methods in patients with pneumonia in a recent local study [2]. Assay development and validation were carried out according to recent guidance [16]. The *lytA* target was chosen for *S. pneumoniae* because it is well characterized, sensitive and specific for the identification of *S. pneumoniae*, and superior to other targets, such as *ply* [17,18]. The commonly used protein D-encoding gene *hpd*#3 assay target for *H. influenzae* was not employed in this study, owing to the known cross-reaction with *Haemophilus aphrophilus* [19]. The *fucK* target was chosen instead, because it had similar sensitivity for the detection of *H. influenzae* and discrimination from *Haemophilus haemolyticus*, and also performed slightly better than *hpd*#3 in a recent reference laboratory-based study [20,21]. The *nuc* target was chosen for *Staphylococcus aureus* detection because it has also been well evaluated in a reference laboratory setting [22]. Use of the *copB* gene target is widespread for the detection of *M. catarrhalis*, but some assays have primer mismatches with published *M. catarrhalis copB* sequences, so an assay in a more conserved region of the gene was chosen [23]. For the detection of *E. coli* and *K. pneumoniae*, assays targeting *yccT* and *gltA*, respectively, were used [24]. Owing to the high level of homology between *E. coli* and *Shigella* species, it was not possible to find an *E. coli*-specific assay target. However, as *Shigella* species are very unlikely to be found in the respiratory tract, this was not deemed to be clinically relevant in the context of LRTI diagnosis. The *gyrB* target was chosen for the detection of *P. aeruginosa* rather than the commonly used *ecfX* target, because of its slightly higher detection rate and specificity on clinical isolates [25–27]. The $bla_{OXA\text{-}51}$-like gene chosen for *A. baumannii* detection has been found in *A. baumannii* isolates, with a few exceptions, and has not been detected in other *Acinetobacter* species [28–30].

Estimation of PCR efficiency, linearity, and quantitative range

Amplification was linear and efficient for all targets within the quantitative range 60–6 000 000 gene copies/reaction, with the exception of the *gyrB* target, which had a quantitative range of 600–6 000 000 gene copies/reaction (Table 4). Within this range, all replicates tested were positive, with Cq value standard deviations of 0.34–2.29 and coefficients of variation of 1.68–8.85% for all targets. Outside the quantitative range, assays were non-linear and less efficient, and low-concentration replicates were not consistently detected.

TABLE 4. Analytical sensitivity and limit of detection; assay slope, linearity and efficiency were calculated for a quantitative range of 60–6 000 000 gene copies/reaction for all targets except for *Pseudomonas aeruginosa*, which had a quantitative range of 600–6 000 000 gene copies/reaction

					Number of replicates (%) detected for input bacterial spike, CFUs/reaction					
		Slope	Linearity (R^2)	Efficiency	100 000 CFUs/PCR (8 333 333 CFUs/mL)	1000 CFUs/PCR (83 333 CFUs/mL)	100 CFUs/PCR (8333 CFUs/mL)	10 CFUs/PCR (833 CFUs/mL)	1 CFU/PCR (83 CFUs/mL)	Limit of detection (95% level), CFUs/reaction
mRT-PCR 1	*Streptococcus pneumoniae*	−3.18	0.92	1.06	12/12 (100)	12/12 (100)	12/12 (100)	12/12 (100)	2/9 (22)	2.1
	Haemophilus influenzae	−3.11	0.97	1.10	12/12 (100)	12/12 (100)	12/12 (100)	12/12 (100)	12/12 (100)	0.8
	Staphylococcus aureus	−3.33	0.98	1.00	12/12 (100)	12/12 (100)	12/12 (100)	7/9 (78)	7/9 (78)	15.5
	Moraxella catarrhalis	−3.18	0.92	1.06	12/12 (100)	12/12 (100)	12/12 (100)	6/12 (50)	3/12 (25)	23.1
mRT-PCR 2	*Escherichia coli*	−3.27	0.95	1.05	12/12 (100)	12/12 (100)	11/11 (100)	10/12 (83)	8/12 (67)	18.5
	Klebsiella pneumoniae	−3.43	0.93	1.00	12/12 (100)	12/12 (100)	11/11 (100)	12/12 (100)	8/12 (67)	1.5
	Pseudomonas aeruginosa	−3.46	0.96	0.96	12/12 (100)	12/12 (100)	10/11 (91)	0/12 (0)	0/12 (0)	104.6
	Acinetobacter baumannii	−3.20	0.96	1.08	12/12 (100)	12/12 (100)	11/11 (100)	12/12 (100)	5/12 (42)	2.1
Control	Human	−3.37	0.99	0.98	NA	NA	NA	NA	NA	NA

mRT-PCR, multiplex real-time PCR; NA, not assessed.

Analytical sensitivity

Analytical sensitivity and limit of detection were estimated with nine to 12 replicates of sputum specimens spiked with bacteria at five different concentrations, from 1 to 100 000 CFUs/reaction (Table 4). Both the mRT-PCR 1 assay and the mRT-PCR 2 assay were highly sensitive for the detection of all bacteria in mock specimens down to the level of 100 CFUs/reaction, except for *P. aeruginosa*, for which the assays were approximately 1 log less sensitive. Limits of detection were approximately 1–100 CFUs/reaction (corresponding to 83–8333 CFUs/mL), depending on the target.

Analytical specificity

Coverage of a large panel of control isolates was 100% for all assay targets, and there were no cross-reactions between targets within the mRT-PCR 1 assay and the mRT-PCR 2 assay. Specificity against a panel of 88 isolates closely related to the target organisms and/or commonly found in the respiratory tract as pathogens or commensals was 100% for all targets, with the exception of *copB*, which was 98% specific, owing to cross-reactivity with *Moraxella lacunata* and *Moraxella nonliquefaciens* (Tables 5 and 6). However, the *copB* assay did not detect *Moraxella osloensis* or *Moraxella atlantae*.

Precision (repeatability)

Precision was measured by comparing bacterial load quantification by the mRT-PCR 1 assay and the mRT-PCR 2 assay for 11–12 replicates of PCR-negative sputum specimens spiked with each of the eight target bacteria at high (100 000 CFUs/reaction), medium (1000 CFUs/reaction) and low (100 CFUs/reaction) concentrations. The numbers of replicates with bacterial load quantified within a 1 log CFUs/reaction range for high, medium and low target concentrations were 96 of 96 (100%), 89 of 96 (92.7%), and 86 of 92 (93.5%), respectively. The most precise assays were for *S. pneumoniae* and *H. influenzae*, because all replicates were within a 1 log CFUs/reaction range. The least precise assays were for *Staphylococcus aureus*, *M. catarrhalis*, and *P. aeruginosa*; these quantified 33 of 36 (91.7%), 32 of 36 (88.9%) and 32 of 35 (91.4%) replicates, respectively, within the 1 log range. Overall, the results indicated that reliable bacterial load quantification from sputum specimens was achievable with both assays down to a concentration of 100 CFUs/reaction, which is equivalent to 8333 CFUs/mL.

Detection of targets in unequal triple mixtures

The Cq value for each target in a mixture was similar to the Cq value for that target alone, and all values were tightly clustered, with standard deviations within the range 0.14–1.10 (Fig. 1). Therefore, all components within a triple mixture, including the minority component, could be accurately

TABLE 5. Analytical specificity summary for multiplex real-time PCR 1 detection (no. (%))

Isolates	*Streptococcus pneumoniae*	*Haemophilus influenzae*	*Staphylococcus aureus*	*Moraxella catarrhalis*
Streptococcus pneumoniae	36/36 (100)	0/36 (0)	0/36 (0)	0/36 (0)
Haemophilus influenzae	0/47 (0)	47/47 (100)	0/47 (0)	0/47 (0)
Staphylococcus aureus	0/41 (0)	0/41 (0)	41/41 (100)	0/41 (0)
Moraxella catarrhalis	0/26 (0)	0/26 (0)	0/26 (0)	26/26 (100)
Panel of 88 respiratory and related organisms + *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*	0/92 (0)	0/92 (0)	0/92 (0)	2/92[a] (2)

[a]*Moraxella lacunata* and *Moraxella nonliquefaciens* were *copB* positive.

TABLE 6. Analytical specificity summary for multiplex real-time PCR 2 detection (no. (%))

Isolates	*Escherichia coli*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Acinetobacter baumannii*
Escherichia coli	28/28 (100)	0/28 (0)	0/28 (0)	0/28 (0)
Klebsiella pneumoniae	0/28 (0)	28/28 (100)	0/28 (0)	0/28 (0)
Pseudomonas aeruginosa	0/26 (0)	0/26 (0)	26/26 (100)	0/26 (0)
Acinetobacter baumannii	0/17 (0)	0/17 (0)	0/17 (0)	17/17 (100)
Panel of 88 respiratory and related organisms + *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Staphylococcus aureus* and *Moraxella catarrhalis*	0/92 (0)	0/92 (0)	0/92 (0)	0/92 (0)

quantified despite the presence of other components at different concentrations.

Testing of clinical specimens

Twenty consecutive culture-negative sputum specimens obtained from individual patients with pneumonia were retrospectively tested with the novel assays. Specimens had a median *GAPDH* concentration of 139 659 gene copies/reaction (range: 10 853–1 030 000 gene copies/reaction). Twelve of 20 (65%) specimens were positive with the mRT-PCR 1 and mRT-PCR 2 assays, with seven (58.3%) single infections and five (41.7%) mixed infections (Table 7). In addition, a further 20 consecutive sputum specimens, culture positive for any of the eight bacterial pathogens targeted by the mRT-PCR 1 and mRT-PCR 2 assays, were retrospectively tested with the novel assays. These specimens had a median *GAPDH* concentration of 134 000 gene copies/reaction (range: 4876–729 000 gene copies/reaction), which was not significantly different from that in the culture-negative group (p 0.968). In 20 of 20 (100%) sputum specimens, the mRT-PCR 1 and mRT-PCR 2 assays detected the same bacterial species grown in culture (Table 7). Furthermore, the novel PCR assays detected an additional 15 bacterial infections, giving a total of 11 (55%) single infections and nine (45%) mixed infections. By semiquantitative sputum culture, the majority of isolates present were reported as large numbers of organisms, corresponding to between 4.68×10^4 CFUs/mL and 1.10×10^{10} CFUs/mL as measured by the mRT-PCR assay (Table 7). However, bacterial loads were significantly lower in the culture-negative group (median: 3.50×10^5 CFUs/mL) than in the culture-positive group (median: 3.33×10^8 CFUs/mL) (p 0.0001) (Fig. 2). Finally, 20 consecutive BAL fluid specimens obtained from individual patients admitted to critical care were retrospectively tested with the novel assays (Table 7). *GAPDH* was quantified in 18 of 20 BAL fluid specimens, and this gave a median *GAPDH* concentration of 24 720 gene copies/reaction (range: 892–176 356 gene copies/reaction), which was significantly lower than in sputum specimens (p 0.0006). Concordant results for bacterial targets in the mRT-PCR assays were achieved for 18 of 20 specimens, with seven additional bacterial infections being identified by PCR. Two specimens gave discordant results for bacterial targets present in the mRT-PCR assays: one specimen grew 3.0×10^2 CFUs/mL of *S. aureus* and was PCR negative; a second specimen grew 1.0×10^4 CFUs/mL of *K. pneumoniae* and was *K. pneumoniae* PCR negative at just below the 100 CFUs/reaction cut-off for the assay. When culture-positive specimens were detected by the mRT-PCR assays, quantitative PCR bacterial loads were in the same order of magnitude as or higher than those of quantitative culture (Table 7). *Candida* species and *Morganella morganii* were identified in five specimens, but targets for these organisms were not present in the PCR assays. In three specimens, overgrowth of respiratory flora is likely to have masked the existence of respiratory pathogens; culture results were reported as 'no significant growth' or 'upper respiratory tract flora', but the mRT-PCR assays identified $>10^4$ CFUs/mL of at least one pathogen.

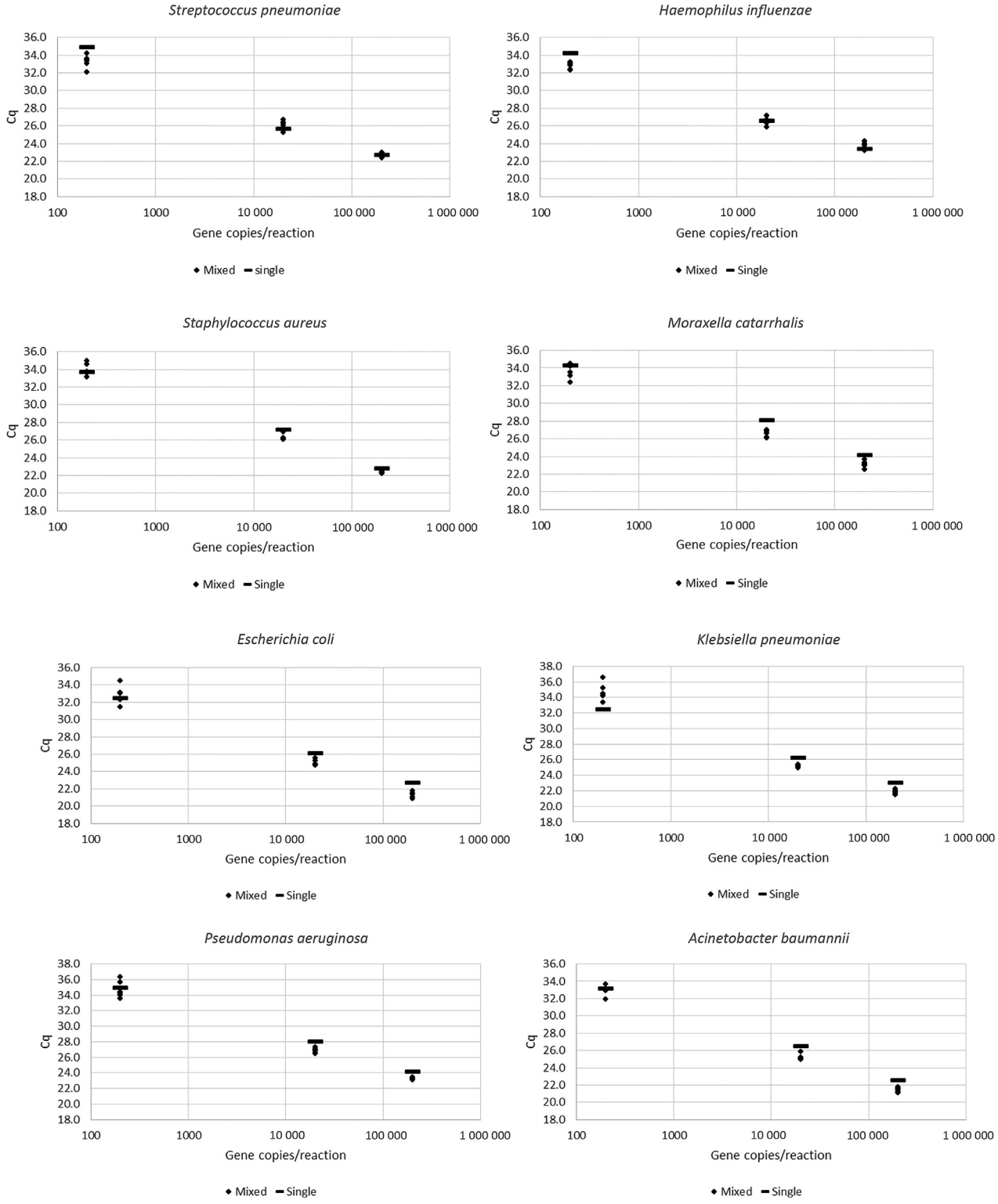

FIG. 1. Distribution of Cq values in triple unequal mixture (n = 6) and single (n = 1) plasmid positive control material for each assay target at three concentrations (200, 20 000 and 200 000 gene copies/reaction).

TABLE 7. Bacterial detection by multiplex real-time PCR (mRT-PCR) 1 and mRT-PCR 2 assays in lower respiratory tract specimens (sputa, $n = 40$; bronchoalveolar lavage (BAL), $n = 20$)

Specimen type (no. of specimens)	Standard culture	mRT-PCR 1 and mRT-PCR 2 result
Sputum	No growth	*Haemophilus influenzae* 2.81×10^6 CFUs/mL
Sputum	No growth	*H. influenzae* 5.56×10^5 CFUs/mL
Sputum	No growth	*H. influenzae* 6.31×10^7 CFUs/mL
Sputum	No growth	*H. influenzae* 3.43×10^5 CFUs/mL
Sputum	No growth	*Streptococcus pneumoniae* 3.87×10^7 CFUs/mL
Sputum	No growth	*S. pneumoniae* 4.26×10^7 CFUs/mL
Sputum	No growth	*Escherichia coli* 1.91×10^5 CFUs/mL
Sputum	No growth	*H. influenzae* 1.47×10^8 CFUs/mL *S. pneumoniae* 1.99×10^4 CFUs/mL
Sputum	No growth	*H. influenzae* 8.42×10^6 CFUs/mL *Moraxella catarrhalis* 1.46×10^4 CFUs/mL
Sputum	No growth	*H. influenzae* 3.50×10^5 CFUs/mL *E. coli* 4.18×10^4 CFUs/mL
Sputum	No growth	*H. influenzae* 9.76×10^3 CFUs/mL *S. pneumoniae* 1.43×10^6 CFUs/mL *M. catarrhalis* 8.74×10^3 CFUs/mL
Sputum	No growth	*S. pneumoniae* 1.12×10^7 CFUs/mL *E. coli* 3.11×10^4 CFUs/mL
Sputum (eight specimens)	No growth	Negative
Sputum	*H. influenzae* (LN)	*H. influenzae* 2.52×10^9 CFUs/mL
Sputum	*H. influenzae* (LN)	*H. influenzae* 1.14×10^9 CFUs/mL
Sputum	*H. influenzae* (LN)	*H. influenzae* 4.06×10^8 CFUs/mL
Sputum	*H. influenzae* (LN)	*H. influenzae* 5.48×10^8 CFUs/mL
Sputum	*H. influenzae* (LN)	*H. influenzae* 5.10×10^9 CFUs/mL
Sputum	*H. influenzae* (LN)	*H. influenzae* 3.24×10^8 CFUs/mL
Sputum	*H. influenzae* (SN)	*H. influenzae* 2.49×10^8 CFUs/mL *S. pneumoniae* 2.47×10^6 CFUs/mL
Sputum	*H. influenzae* (LN)	*H. influenzae* 3.41×10^8 CFUs/mL *S. pneumoniae* 1.58×10^8 CFUs/mL
Sputum	*H. influenzae* (LN)	*H. influenzae* 1.10×10^{10} CFUs/mL *M. catarrhalis* 2.22×10^4 CFUs/mL
Sputum	*H. influenzae* (LN)	*H. influenzae* 4.68×10^4 CFUs/mL *M. catarrhalis* 9.42×10^6 CFUs/mL
Sputum	*H. influenzae* (LN)	*H. influenzae* 1.77×10^9 CFUs/mL *S. pneumoniae* 3.79×10^4 CFUs/mL *M. catarrhalis* 2.45×10^4 CFUs/mL *Staphylococcus aureus* 3.49×10^4 CFUs/mL
Sputum	*S. pneumoniae* (LN)	*S. pneumoniae* 8.67×10^8 CFUs/mL
Sputum	*S. pneumoniae* (LN)	*S. pneumoniae* 1.65×10^7 CFUs/mL *H. influenzae* 9.42×10^3 CFUs/mL *M. catarrhalis* 4.43×10^4 CFUs/mL
Sputum	*S. pneumoniae* (LN)	*S. pneumoniae* 1.12×10^5 CFUs/mL *H. influenzae* 6.28×10^8 CFUs/mL *M. catarrhalis* 1.42×10^7 CFUs/mL *E. coli* 2.95×10^6 CFUs/mL *Klebsiella pneumoniae* 2.36×10^5 CFUs/mL
Sputum	*E. coli* (LN)	*E. coli* 8.92×10^8 CFUs/mL
Sputum	*E. coli* (LN)	*E. coli* 1.17×10^5 CFUs/mL *S. pneumoniae* 1.42×10^4 CFUs/mL
Sputum	*Pseudomonas aeruginosa* (MN)	*P. aeruginosa* 8.27×10^4 CFUs/mL
Sputum	*P. aeruginosa* (LN)	*P. aeruginosa* 6.02×10^6 CFUs/mL *S. pneumoniae* 1.97×10^7 CFUs/mL
Sputum	*M. catarrhalis* (LN)	*M. catarrhalis* 2.40×10^8 CFUs/mL
Sputum	*Staphylococcus aureus* (MN)	*Staphylococcus aureus* 1.59×10^7 CFUs/mL
BAL (seven specimens)	No growth	Negative
BAL	No growth	*H. influenzae* 9.71×10^3 CFUs/mL
BAL	No significant growth	*H. influenzae* 5.07×10^6 CFUs/mL
BAL	No significant growth	*E. coli* 1.29×10^5 CFUs/mL *Staphylococcus aureus* 1.85×10^4 CFUs/mL
BAL	Upper respiratory tract flora *Candida albicans* 2×10^2 CFUs/mL	*S. pneumoniae* 4.30×10^5 CFUs/mL
BAL	*Candida albicans* 7×10^3 CFUs/mL	Negative
BAL	*Candida glabrata*	Negative
BAL	*Morganella morganii* 1×10^4 CFUs/mL	Negative
BAL	*E. coli* 2×10^3 CFUs/mL *S. pneumoniae* 1×10^4 CFUs/mL *H. influenzae* 1×10^4 CFUs/mL	*E. coli* 3.31×10^5 CFUs/mL *S. pneumoniae* 2.90×10^6 CFUs/mL *H. influenzae* 6.21×10^7 CFUs/mL
BAL	*P. aeruginosa* $>1 \times 10^4$ CFUs/mL	*P. aeruginosa* 2.21×10^5 CFUs/mL
BAL	*E. coli* $>1 \times 10^4$ CFUs/mL	*E. coli* 1.48×10^8 CFUs/mL (*M. catarrhalis* target just below cut-off)
BAL	*E. coli* 3×10^2 CFUs/mL *Candida tropicalis* $>1 \times 10^4$ CFUs/mL	*E. coli* 8.38×10^5 CFUs/mL *H. influenzae* 3.26×10^5 CFUs/mL
BAL	*Staphylococcus aureus* 3×10^2 CFUs/mL	Negative
BAL	*K. pneumoniae* 1×10^4 CFUs/mL	*Staphylococcus aureus* 5.71×10^4 CFUs/mL (*K. pneumoniae* target just below cut-off)

LN, large numbers; MN, moderate numbers; SN, small numbers.

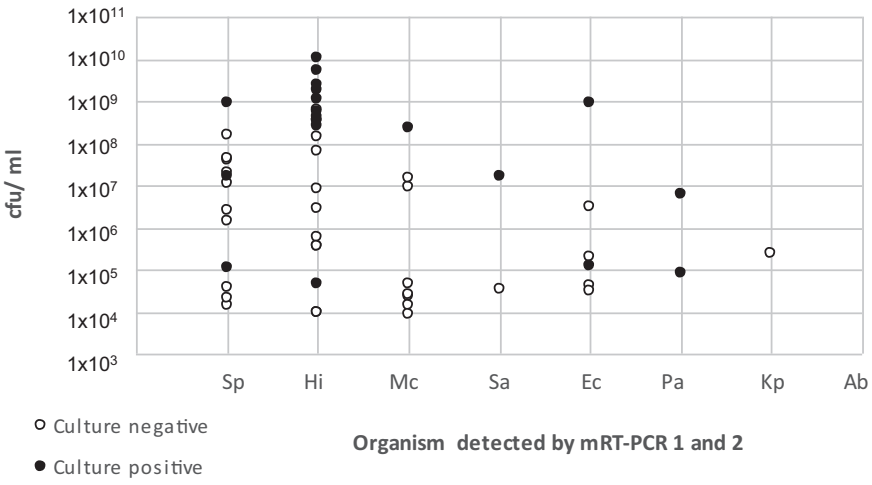

FIG. 2. Bacterial loads calculated by multiplex real-time PCR (mRT-PCR) in culture-positive (n = 20) and culture-negative (n = 20) sputum specimens from patients with pneumonia. Ab, *Acinetobacter baumannii*; Ec, *Escherichia coli*; Hi, *Haemophilus influenzae*; Kp, *Klebsiella pneumoniae*; Mc, *Moraxella catarrhalis*; Pa, *Pseudomonas aeruginosa*; Sa, *Staphylococcus aureus*; Sp, *Streptococcus pneumoniae*.

Discussion

We have described the development and validation of two quantitative mRT-PCR assays for eight important respiratory bacterial pathogens, and demonstrated their potential as an improved diagnostic tool in LRTI. Through *in silico* analysis and *in vitro* experimentation, we have designed assays that sensitively and specifically detect the targeted bacterial species. Bacterial targets could be reliably quantified from sputum specimens down to a concentration of 100 CFUs/reaction or approximately 8000 CFUs/mL.

A key strength of our study was that all 249 positive control isolates were correctly detected with our assay, demonstrating its effectiveness on both reference strains and local clinical isolates. Concordance with culture for 20 culture-positive sputum specimens was also 100%. In addition, specificity on a panel of nearly 100 negative control isolates was 98%. Furthermore, the two assays quantified bacterial load accurately when three bacterial targets were present in mixtures of varying concentrations, mimicking likely clinical scenarios in LRTI. These assays are combined with controls for specimen quality and reaction inhibition, and run on a standard fast real-time PCR platform, enabling results to be produced within 6 h of specimen receipt. To the best of our knowledge, there are no other quantitative molecular assays for LRTI diagnosis that have been as extensively validated and meet these criteria.

One of the weaknesses of our study was that an extended extraction protocol was required before processing on an automated platform, increasing manual complexity and turn-around time. This was necessary for efficient extraction of *Staphylococcus aureus* DNA from sputum specimens, as we had previously found that mock specimens containing *Staphylococcus aureus* spikes were underquantified with standard automated extraction only. Enzymatic lysis of *Staphylococcus aureus* with achromopeptidase has been described as both rapid and efficient, and therefore warrants further investigation as an alternative method in this setting [31]. However, when particularly mucopurulent specimens, such as sputa, are used, an additional proteinase K incubation step may still be required, in order to fully break down the material before addition to an automated extraction system. Despite the limitations of the current protocol, results are achievable in 6 h, owing to the use of automated systems and fast mRT-PCR, thus enabling same-day reporting for specimens received in the morning. This is still quicker than culturing even the faster-growing Gram-negative pathogens in our panel, and means that a comprehensive multi-pathogen result is available at a single time-point.

We set criteria for acceptance of quantification results based on the Cq of the internal control falling within a 1 log range; outside of this range, quantitative results were not accepted, owing to the potential for partial PCR inhibition leading to inaccuracy of measurement. However, as our quantitative mRT-PCR and internal control assays utilize different reagents, they may not be equally affected by the presence of PCR inhibitors, and therefore a bacterial load output from an assay meeting the quality control criteria could still potentially be an underestimate. This was not an issue during the sputum bacterial spiking experiments carried out here, although these were limited in number. Similarly, testing of BAL fluid specimens showed that quantitative PCR bacterial loads were generally higher than those obtained with quantitative culture, but BAL fluid is significantly less cellular than sputum, and would therefore be expected to be less inhibitory.

Furthermore, we tested only a small number of sputum and BAL specimens for comparison with culture in a proof-of-principle experiment. However, this demonstrated the potential clinical utility of our molecular assays and the suitability of

these two contrasting LRT specimen types. It was clear from our small collection of clinical specimens that there is an added benefit with mRT-PCR; it can detect more bacterial infections than culture alone, and provide a simultaneous quantitative output. Specimens that were culture positive for any of the eight bacterial pathogens targeted by the mRT-PCR assays had higher bacterial loads than those that were culture negative, indicating the increased sensitivity of PCR as compared with culture. This may prove to be particularly useful in the hospital setting, where patients are more likely to have prior antibiotic exposure before specimens are taken, thus reducing bacterial burden and viability.

A significant issue that we discovered during development was that the *copB* assay for *M. catarrhalis* cross-reacted with *M. nonliquefaciens* and *M. lacunata* isolates. Although several other authors have used assays based on the *copB* gene to target *M. catarrhalis* [23,32–34], none have reported its specificity against *M. nonliquefaciens*, and only one study tested *M. lacunata*, with no cross-reactivity being detected [32]. *In silico* analysis did not predict *copB* cross-reactions, because of a lack of genomic sequence data and *copB* gene sequences for *Moraxella* species in GenBank. *M. nonliquefaciens* and *M. lacunata* are members of the normal respiratory microbiota, but rarely cause respiratory tract infection themselves [35]. The paucity of sequence data makes design of a more specific *M. catarrhalis* assay problematic, and therefore the clinical relevance of this cross-reaction is uncertain at present. However, the flexibility of an in-house mRT-PCR format means that, once further sequence information is available, a modified *M. catarrhalis* assay could be readily incorporated and revalidated. In the meantime, we recommend that other groups who currently use the *copB* gene target for *M. catarrhalis* be aware of the potential for cross-reactivity with some *Moraxella* species when reporting results from direct detection on respiratory specimens. Furthermore, as the *copB* assay did not cross-react with *M. osloensis* or *M. atlantae*, this assay cannot be regarded as a genus-specific *Moraxella* species assay. Cross-reactivity is also an issue for *E. coli* PCR assays, owing to the high level of homology between *E. coli* and *Shigella* species. However, as *Shigella* species are very unlikely to be found in the respiratory tract, this is unlikely to be diagnostically relevant in a respiratory context.

In order to enable accurate molecular diagnosis of LRTI, a specimen should ideally be: (a) obtained from the LRT rather than the upper respiratory tract (URT); and (b) of good quality, i.e. purulent material rather than salivary in the case of sputum. This is because of the potential for detection of colonization rather than infection when URT specimens are used, and the potential for contamination of LRT specimens with oral flora during sampling. For this reason, our assays have been developed for use on sputum and BAL fluid specimens rather than throat swabs or nasopharyngeal aspirates, and have an in-built measure of cellular content for quality control (*GAPDH*). Microscopy can be carried out to determine the quality of a sputum specimen based on the numbers of polymorphonuclear leukocytes and squamous epithelial cells [1], but *GAPDH* is present in all cells, and therefore does not allow such discrimination. Sputum microscopy is not routinely carried out in our centre, so we do not know to what extent our cellular load outputs were likely to be influenced by contaminating epithelial cells from the URT, but we deliberately used only macroscopically purulent material from sputum specimens for PCR in this study for a direct comparison with culture.

Rates of URT asymptomatic carriage of *S. pneumoniae* and *H. influenzae* in adults have been studied most frequently by PCR, and range from 2% and 39%, depending on the setting [36,37]. Furthermore, molecular studies using paired specimens have shown that the bacteria and viruses detected in the URT may not accurately reflect the organisms detected in the LRT [38–40]. However, quantification of bacterial DNA load may be important in distinguishing infection from asymptomatic colonization; most work described to date has focused on *S. pneumoniae*, and a cut-off of 10^4 to 10^5 gene copies/mL has typically been described [3,41–43]. Therefore, our assay could be used to investigate whether similar clinical cut-offs can be determined for other respiratory bacteria and for conditions other than pneumonia. In comparison with semiquantitative sputum culture in patients with pneumonia, we found that, although the majority of isolates were reported as 'large numbers' of organisms in monomicrobial infections, a wide range of CFUs/mL was measured by quantitative PCR and many organisms were detected in mixed infections in different proportions. In some cases, the organism grown in culture was not the organism with the highest bacterial load in a polymicrobial infection as determined by quantitative PCR. Overall, these observations may indicate the tendency of one organism to overgrow others in culture, leading to under-reporting of mixed infections. Our assay could also be used in settings where quantitative culture of respiratory specimens is the norm and rapid diagnosis is of key importance. For example, the use of quantitative culture for BAL fluid is standard for the microbiological diagnosis of ventilator-acquired pneumonia, with a cut off of 10^4 CFUs/mL [44]. As proof-of-principle, we were able to quantify bacterial loads in BAL fluid specimens from critical-care patients with comparable results to those obtained with culture, and many of the major ventilator-acquired pneumonia pathogens are included in our assay. Therefore, the use of mRT-PCR assays could provide a faster quantitative result in this setting. Although not validated for use here, there is also scope to use our mRT-PCR assays on whole

blood specimens, as higher DNA loads have been found to correlate with disease severity in some studies [45–47].

In conclusion, a quantitative molecular test for the key bacterial causes of LRTI has the potential to provide a more sensitive decision-making tool, closer to the time-point of patient admission, than current standard methods. A faster and more accurate microbiological diagnosis should facilitate de-escalation from broad-spectrum to narrow-spectrum antibiotics, substantially improving patient management and supporting efforts to curtail inappropriate antibiotic use.

Transparency declaration

The authors have no conflicts of interest to declare for this work.

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