Bacteriology

Quantitative detection of *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* in lower respiratory tract samples by real-time PCR

Madeleine Kais\textsuperscript{a}, Carl Spindler\textsuperscript{b,c}, Mats Kalin\textsuperscript{b,c}, Åke Örtqvist\textsuperscript{d}, Christian G. Giske\textsuperscript{a,*}

\textsuperscript{a}Clinical Microbiology, Microbiology and Tumor Biology Center, Karolinska Institutet, Karolinska University Hospital Solna, SE-17176 Stockholm, Sweden
\textsuperscript{b}Unit of Infectious Diseases, Department of Medicine, Karolinska Institutet, SE-17176 Stockholm, Sweden
\textsuperscript{c}Department of Infectious Diseases, Karolinska University Hospital Solna, SE-17176 Stockholm, Sweden
\textsuperscript{d}Department of Communicable Diseases Control and Prevention, Stockholm County, SE-17176 Stockholm, Sweden

Received 22 August 2005; accepted 3 January 2006

Abstract

The limitation of polymerase chain reaction (PCR) in diagnosis of lower respiratory tract infections (LRTIs) caused by *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* has been a distinguishing colonization from infection. We assess here the usefulness of real-time quantitative PCR (RQ-PCR) performed on lower respiratory tract samples to overcome this problem.

Consecutive respiratory tract samples from patients with and without signs of infection (\(n = 203\)) were subjected to RQ-PCR, targeting the genes pneumolysin (\(S. pneumoniae\)), fumarate reductase (\(H. influenzae\)), and outer membrane protein B (\(M. catarrhalis\)). DNA from positive controls with predefined colony forming units (CFUs) per milliliter were included to allow estimation of CFU per milliliter for the test samples. In parallel, assessment of quantitative cultures from all samples was performed.

In the group of patients with LRTI, significant pathogens (\(\geq 10^5\) CFU/mL) were found in 32/135 samples (23.7%) with culture, in 51/135 (37.7%) with RQ-PCR, and in 59/135 (43.7%) when combining the methods.

\(© 2006\) Elsevier Inc. All rights reserved.

Keywords: Community-acquired pneumonia; Etiology; Diagnostic methods

1. Introduction

Respiratory tract infection, mainly in the form of pneumonia, is an important cause of morbidity and mortality, especially in children and the elderly (Hedlund and Örtqvist, 2002). Establishing a microbiologic diagnosis is considered an important measure for a proper treatment, but even in the most rigorous studies, it is difficult to establish an etiologic diagnosis in 50% of cases of community-acquired pneumonia (CAP) (Reimer and Carroll, 1998). The most frequently detected pathogens are *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Mycoplasma pneumoniae* (File, 2003). Conflicting evidence exists regarding the importance of pathogens such as *Moraxella catarrhalis*, and the high rates of colonization has lead to a general skepticism toward the role of this pathogen (Reimer and Carroll, 1998; Karalus and Campagnari, 2000), though some reports suggest an important role for this bacterium in selected patient groups, such as patients with chronic obstructive pulmonary disease (COPD) (Karakus and Campagnari, 2000; Hedlund et al., 1997).

Lower respiratory tract cultures have so far been the most important method for diagnosing respiratory tract infections. Recently, immunochromatographic urinary antigen detection assays for pathogens such as *S. pneumoniae* and *Legionella pneumophila* have shown promising results as supplementary tests with relatively high sensitivities and specificities (Dominguez et al., 1999; Murdoch et al., 2001). However, the need for still more sensitive methods has lead to studies evaluating the role of nucleic acid amplification methods such as polymerase chain reaction (PCR) (Murdoch, 2003). For pathogens such as *S. pneumoniae*,

* Corresponding author. Department of Clinical Microbiology, Karolinska University Hospital, SE-17176 Stockholm, Sweden. Tel.: +46-8-517-73574; fax: +46-8-30-80-99.
E-mail address: christian.giske@karolinska.se (C.G. Giske).

0732-8893/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.diagmicrobio.2006.01.007
Chlamydia pneumoniae, and L. pneumophila, PCR is already available (Welti et al., 2003). However, for S. pneumoniae, H. influenzae, and M. catarrhalis, which are frequent colonizers, the main challenge with regular PCR assays has been the difficulties in distinguishing colonization from infection because these assays do not generate quantitative data (Murdoch, 2004).

Potentially, this obstacle could be overcome by the use of a quantitative PCR assay. The diagnostic properties of real-time quantitative PCR (RQ-PCR) have been evaluated in a few studies of both upper and lower respiratory tract samples. Two studies on the detection of S. pneumoniae in nasopharyngeal secretion by targeting the pneumolysin gene showed high sensitivity, specificity, and reproducibility, as well as good correlation with quantitative cultures (Greiner et al., 2001; Saukkoriipi et al., 2004). Similar conclusions could be drawn from a study of the detection of M. catarrhalis in nasopharyngeal secretions (Greiner et al., 2003). Two recent studies have also evaluated RQ-PCR for the detection of S. pneumoniae in sputum and bronchoalveolar lavage fluid (BAL) (Apfalter et al., 2005; Yang et al., 2005). In both studies, the novel assays were found to yield rapid and accurate diagnosis of pneumococcal pneumonia.

Although RQ-PCR has been applied on lower respiratory tract samples for the detection of S. pneumoniae, no available assay exists for multiple traditional pathogens. We present here a comparison of the properties of quantitative culture and a novel RQ-PCR assay for quantitative detection of S. pneumoniae, H. influenzae, and M. catarrhalis in lower respiratory tract samples.

2. Materials and methods

2.1. Bacterial strains

S. pneumoniae CCUG 28588, M. catarrhalis CCUG 18284, and H. influenzae CCUG 23969 (Culture Collection, University of Gothenburg, Sweden) were used as positive controls.

Specificity of the real-time PCR assay for S. pneumoniae was tested with reference strains Streptococcus anginosus CCUG 27298, Streptococcus milleri NCTC 10708 (National Collection of Type Cultures, London, UK), Streptococcus mitis ATCC 15912 (American Type Culture Collection, Manassas, VA), as well as the strains Streptococcus sanguis 804, Streptococcus intermedius 75:5, Streptococcus constellatus 90:7, and Streptococcus mutans 1B (all derived from Karolinska University Hospital, Huddinge, Sweden). Lastly, 5 clinical isolates of Peptostreptococcus spp. were also included in the specificity analysis.

Specificity of the real-time PCR assay for H. influenzae and M. catarrhalis was tested with the reference strains Neisseria lactamica CCUG 5853, Bordetella pertussis CCUG 35196, Haemophilus parainfluenzae CCUG 12836, Neisseria meningitidis TCC 13077, Staphylococcus aureus ATCC 29213, and S. pneumoniae ATCC 33400. Furthermore, a collection of clinical isolates derived from Karolinska University Hospital, Solna, Sweden, were tested. The collection consisted of 1 isolate of Moraxella nonliquefaciens and S. milleri, 2 isolates of Pasteurella multocida and Moraxella subsp. moraxella, 3 isolates of Haemophilus parahaemolyticus, and 7 isolates of H. parainfluenzae.

2.2. Clinical samples

A total of 203 consecutive respiratory tract samples (106 sputum, 51 protected specimen brush [PSB], and 46 BALs) obtained from the routine diagnostic laboratory at Karolinska University Hospital during October 2004 were examined with the RQ-PCR assay. Clinical data were collected retrospectively, by review of clinical charts. For 14 patients (6.9%), all of them from intensive care units, proper data could not be obtained. The clinical material consisted of 89 cases of CAP, 27 cases of nosocomial pneumonia, 19 cases of bronchitis or acute exacerbation of COPD, as well as 54 cases with various noninfectious pulmonary conditions, such as suspected lung tumor or fibrosis. Pneumonia was defined on the basis of clinical suspicion by the patient’s physician. As displayed in Table 1, the material was heterogeneous, as would be expected when collecting a consecutive clinical material from a microbiologic laboratory.

Throat swabs from 10 healthy laboratory workers were also used in the specificity analysis of the real-time PCR assay for the 3 pathogens.

The study was approved by the local human investigations committee.

2.3. Quantitative respiratory culture

Sputum samples were homogenized with an equal volume of Sputolysin (Calbiochem-Behring, La Jolla, CA) on a vortex mixer and allowed to stand for 20 min. With disposable plastic inoculators, 10 μL of the suspension was diluted 1:100 in brain heart infusion-hemin-iso vitalex-albumin (BHI-HIA) broth and spread onto plates containing selective media. All sputum samples were examined by microscopy, and samples containing a preponderance of leukocytes and few squamous epithelial cells according to accepted criteria (Bartlett et al., 2000) were considered acceptable. Samples containing fewer leukocytes were considered unacceptable and were excluded from further analysis. After 48 h of incubation, colonies were counted.

Table 1

<table>
<thead>
<tr>
<th>Demographic variable</th>
<th>Number of cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAP (clinical definition)</td>
<td>89/189 (47.1)</td>
</tr>
<tr>
<td>Hospital-acquired pneumonia</td>
<td>27/189 (14.3)</td>
</tr>
<tr>
<td>Bronchitis or exacerbation of COPD</td>
<td>19/189 (10.1)</td>
</tr>
<tr>
<td>Noninfectious pulmonary disorders</td>
<td>54/189 (28.6)</td>
</tr>
<tr>
<td>Proportion of smokers</td>
<td>47/189 (24.9)</td>
</tr>
<tr>
<td>Proportion of patients with COPD</td>
<td>70/189 (37.0)</td>
</tr>
<tr>
<td>Antibiotic treatment before sampling</td>
<td>35/189 (18.5)</td>
</tr>
</tbody>
</table>

* Clinical data not available for 14/203 patients (6.9%).
and colony forming units (CFUs) per milliliter calculated (Kalin and Lindberg, 1983b, Murray et al., 2003).

For PSB samples, 1 µL of bronchial secretion was diluted 1:1000 in BHI-HIA broth, and for BAL samples, no dilution was performed. The following procedure, except microscopy, was identical to the procedure applied on sputum samples. To obtain comparable quantitative results, PSB and BAL samples were for the purpose of this study considered as methods for obtaining sputum. The concentration of bacteria was expressed to reflect the concentration in sputum from the lower airways including a dilution factor of 10^3 for PSB and 10^2 for BAL (Baselski and Wunderink, 1994).

All plates were plated on the following aerobic media: blood, chocolate agar, blood agar containing gentiana violet, and cysteine lactose electrolyte-deficient agar. Blood agar plates were also incubated anaerobically. Aerobic plates were first read after 18 h of incubation and then incubated for an additional 24-h period before the final reading. Anaerobic plates were incubated for 48 h. *S. pneumoniae* was identified by colony morphology and optochin susceptibility. *H. influenzae* was identified with morphologic features as well as factor V and X tests. Identification of *M. catarrhalis* was performed with the oxidase test as well as the presence of typical colony morphology and the “hockey puck sign” (Murray et al., 2003).

2.4. DNA extraction

DNA extraction was performed with BioRobot M48 (Qiagen, VWR International, Stockholm, Sweden) according to manufacturers’ instructions. Extracts were stored at −20 °C until required for analysis.

2.5. Quantitative and qualitative PCR

For detection and quantification of *S. pneumoniae*, we used primers specific for pneumolysin gene (*ply*) (Walker et al., 1987; Greiner et al., 2001). Pneumolysin is a species-specific protein toxin produced practically by all clinical isolates of *S. pneumoniae* (Paton et al., 1983) and has been shown to be well conserved (Lock et al., 1996). The nucleotide sequence of the forward primer was 5'-AGC GAT AGC TTT CTC CAA GTG G-3' (position 531 to 552), and the sequence of the reverse primer was 5'-AGC CAA ATC GAT CTT TTA CAA CC-3' (position 583 to 605) with an amplicon of 75 base pairs. (Greiner et al., 2001) Primers were obtained from Cybergene (Huddinge, Sweden).

We also evaluated *atpA* as an alternative species-specific target gene in selected isolates. Culture-based species identification of pneumococci is based on optochin susceptibility, and the molecular correlate for this is the presence of a gene coding for an adenosine triphosphatase that is the target of optochin. (Martín-Galiano et al., 2003) Bacteria lacking this gene, or having a mutated variant, are optochin resistant. The sequence of the *atpA* forward primer was 5'-CGC TAA TTT ACA GTG TGA C-3' (position 783 to 801) and reverse primer 5'-TAA ATC CAC GAC GAC GAA C-3' (position 847 to 865), with an amplicon of 84 base pairs. Primers were designed based on alignment of all available *atpA* sequences in GenBank (www.ncbi.nlm.nih.gov) with the Oligo Primer analysis software, version 6.67 (Molecular Biology Insights, West Cascade, CO).

We used the outer membrane protein gene (*copB*) (Aebi et al., 1998; Greiner et al., 2003) for detection and quantification of *M. catarrhalis*. The nucleotide sequence of the forward primer was 5'-GTG AGT GCC GCT TTA CAA CC-3' (position 50 to 70), and the sequence of the reverse primer was 5'-TGT ATC GCC TGC CAA GAC AA-3' (position 102 to 121), and the amplified fragment was 71 base pairs. Because this gene is regarded as highly specific for *M. catarrhalis*, we did not include additional target genes (Greiner et al., 2003).

For detection and quantification of *H. influenzae*, the housekeeping gene fumarate reductase iron–sulfur gene B (*fjdB*) was chosen. Searches in GenBank indicated a high level of conservation of the gene and low homology with other *Haemophilus* spp. The nucleotide sequence of the forward primer was 5'-ATC GAA AGT TTA TTA GAG GCA A-3' (position 328 to 348), whereas the sequence of the reverse primer was 5'-TTC TTT CGA TGG ATG TGG TT-3' (position 392 to 412). The amplicon was 84 base pairs. For verification of positive findings, a qualitative PCR assay with primers targeting the 16S rRNA gene was performed (Strälin et al., 2005) to be able to amplify a larger fragment than possible in real-time PCR. The sequence of the forward primer was 5'-TCC TAA GAA GAG CTC AGA GAT-3 (position 994 to 1014), and of the reverse primer, 5'-TGA TCC AAC CGC AGG TTC C-3 (position 1513 to 1531). The size of the amplicon was 538 base pairs.

The principle of the RQ-PCR has been described extensively before (Giuilietti et al., 2001). The RQ-PCR assay was performed in Rotor-Gene 3000 (Corbett Research, Mortlake, Sydney, Australia) and analyzed by using a software program from Real-Time Analysis Software (Corbett Research). The real-time PCR amplifications were performed in 20-µL reactions containing 2X QantiTect SYBR Green PCR master mix (Qiagen, VWR international, Huddinge, Sweden), which includes HotStarTaq DNA Polymerase, QantiTect SYBR Green PCR Buffer, 2.5 mmol/L MgCl₂, deoxyribonucleotide triphosphate (dNTP) mix, and fluorescent dyes, RNase-free H₂O (SIGMA; Sigma-Aldrich, Stockholm, Sweden), 0.6 µmol/L primer (Cybergene), and 2 µL of the respective template DNA dilution.

The RQ-PCR assay for *H. influenzae* and *M. catarrhalis* was optimized to an initial activation step of 95°C for 15 min, followed by 40 cycles of denaturation at 94°C for 20 s, annealing at 50°C for 20 s, extension at 72°C for 20 s, followed by a final extension step at 65°C for 30 s. For the RQ-PCR assay of *S. pneumoniae*, the same conditions as for *H. influenzae* and *M. catarrhalis* were used, except an annealing temperature of 60°C for *ply* and of 45°C for *atpA*.

The regular PCR assay was performed in a GeneAmp PCR System 9700 (Applied Biosystems, Stockholm, Sweden) with
1 cycle of 95°C for 10 min, 40 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 10 min. Polymerase chain reaction master mix contained 10 mmol/L Tris–HCl, 50 mmol/L KCl, 2.5 mmol/L MgCl₂, 0.6 U TaqGold (Applied Biosystems), 200 μmol/L dNTPs (Amersham Biosciences, Uppsala, Sweden), and 0.5 μmol/L of each primer in a volume of 23.5 μL. Template DNA was added to a final volume of 25 μL. Amplicons were analyzed by gel electrophoresis (NuSieve 3:1) (BioWhittaker/In vitro Sweden, Stockholm, Sweden).

2.6. Standard curves for positive controls

Positive controls containing known concentrations of template were used for establishing correlation curves between bacterial concentrations and cycle threshold (Cₜ) values in the RQ-PCR. Two different serial dilutions schemes were made: 1 for viable counts and CFU per milliliter calculation, and 1 for DNA quantification with RQ-PCR. A dense suspension of bacteria grown on agar plates was inoculated in phosphate-buffered saline, representing a bacterial concentration of approximately 10⁸ CFU/mL. The concentration of DNA corresponding to 10⁸ CFU/mL was determined spectrophotometrically with the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). Starting from this concentration, a 10-fold serial dilution scheme ranging between 10⁸ and 10³ CFU/mL was prepared. Number of CFUs were determined by plating 10 μL of each dilution step onto agar plates and incubating them at 37°C in 5% CO₂ overnight. The number of colonies was counted after 24 h.

Two hundred microliters of the highest concentration (10⁸ CFU/mL) was used for DNA extraction in BioRobot M48. A standard curve for quantification in the PCR-assay was constructed by using a monoculture of S. pneumoniae, H. influenzae, and M. catarrhalis also in 10-fold serial dilution scheme ranging from 10⁸ to 10³ bacteria per milliliter. The Cₜ values from the PCR assay were plotted against the 10-logarithm of the CFU per milliliter, and a standard curve was generated. A linear correlation between the calculated Cₜ values and the 10-log of CFU per milliliter bacteria was observed.

2.7. Sensitivity, specificity, and amplification efficiency of real-time PCR assay

Standard curve serial dilutions from S. pneumoniae, H. influenzae, and M. catarrhalis were used to determine the sensitivity of the PCR assay, as well as the threshold for the detection of consistent amounts of bacteria between runs. For assessment of specificity of the real-time PCR assay for S. pneumoniae, H. influenzae, and M. catarrhalis, the above-described bacterial strains and the throat swabs were tested against the positive controls.

The amplification efficiency was determined by plotting cycle threshold values against the DNA copy number and calculating the correlation coefficient. A correlation coefficient of 1.00 corresponds to an amplification efficiency of 100%, which translates to a doubling of the amount of DNA after each cycle. Amplification efficiency at 100% indicates logarithmic amplification or an increase in Cₜ values of 3–4 for every 10-log decrease in concentration of DNA.

2.8. Inhibition control for the RQ-PCR assay

Inhibition control for the ply assay was performed on 15 randomly selected negative invasive samples and 15 sputum samples, as well as culture-positive and RQ-PCR-negative samples. For the frdB and copB assays, inhibition control was performed only on culture-positive or RQ-PCR–negative samples. DNA preparations from clinical samples were spiked with extracted DNA from S. pneumoniae CCUG 28588, M. catarrhalis CCUG 18284, and H. influenzae CCUG 23969 to a final concentration corresponding to approximately 25,000 CFU/mL. Real-time quantitative PCR was performed with 2 μL of template DNA, prepared as described above, in 18 μL of reaction volume. Three preparations of positive controls containing only extracted DNA from the respective pathogens were run on 3 separate occasions to generate a mean Cₜ value for each control. Observed Cₜ value for a spiked clinical sample differing more than 2.5 SD from the mean of the positive controls was considered to indicate inhibition.

2.9. Quantification of clinical samples with RQ-PCR

The 203 (106 sputum, 51 PSB, 46 BAL) consecutive respiratory tract samples were tested. For each separate run, 3 positive controls with different concentrations were included to be able to estimate CFU per milliliter from the obtained Cₜ values. This measure was chosen to correct for possible interassay variations in absolute Cₜ values. Real-time quantitative PCR was repeated once in all samples with significant culture and lower amounts of DNA than expected with RQ-PCR.

2.10. Definitions and interpretation of RQ-PCR findings

Significant findings in RQ-PCR were defined as amounts of DNA corresponding to ≥10⁵ CFU/mL. Samples containing amounts of DNA corresponding to less than 10⁴ CFU/mL were considered insignificant, whereas samples with 10⁴ CFU/mL were considered possibly significant. The cutoff values were derived from studies of quantitative sputum cultures related to blood culture results (Kalin and Lindberg, 1983a).

Melting curve analysis was performed on all samples, and temperature acceptance range for the analysis was calculated by averaging the values obtained with control strains for the respective species (mean ± 3 SD). All PCR products displaying melting temperatures outside the acceptance range were regarded nonspecific and hence considered negative.

Statistical analysis of the data was performed with 2-sided t test.
3. Results

3.1. Sensitivity and amplification efficiency of the RQ-PCR assay

Tenfold serial dilutions from a concentration of $10^8$ CFU/mL of *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* were used to construct standard curves for determination of the sensitivity of the real-time PCR assay. The concentration of DNA corresponding to $10^8$ CFU/mL was approximately 40 ng/µL for *S. pneumoniae*, 100 ng/µL for *H. influenzae*, and 200 ng/µL for *M. catarrhalis*. Genomic equivalents (GEQ) per milliliter were calculated for the 3 species and were $1.8 \times 10^9$, $4.7 \times 10^9$, and $9.4 \times 10^9$ GEQ/mL.

The *ply* assay was able to detect bacterial DNA over a linear range between $10^8$ and $10^4$ CFU/mL, with $C_T$ values between 16 and 31. Generally, the *ply* assay generated more accurate quantitative data for the dilution series of the laboratory strains than the *atp* assay, indicating that the *ply* gene was the most suitable target for quantification of *S. pneumoniae* (data not shown). The *frdB* assay detected DNA between $10^8$ and $10^3$ CFU/mL, with $C_T$ values ranging between 13 and 32. Lastly, the *copB* gene assay detected DNA between $10^8$ and $10^3$ CFU/mL, with $C_T$ values ranging from 10 to 28. Standard curves with the $C_T$ values plotted against the 10-log of the CFU per milliliter are shown in Figs. 1–3. The amplification efficiency of the *ply* assay was 86%, for the *frdB* assay 84%, and for the *copB*-assay 84%.

3.2. Specificity of the RQ-PCR

The specificity of the real-time PCR assay was tested with the different bacterial species and the previously described throat samples. Because the specificity of the PCR is temperature dependent, the annealing temperature had to be optimized for the respective microorganisms. Melting curve analysis is another important method for determining the specificity of the PCR products. Accepted temperature range for the melting curve analysis was 79.9–81.2°C for *S. pneumoniae*, 76.0–77.2°C for *H. influenzae*, and 81.5–82.5°C for *M. catarrhalis*.

Evaluation of *ply* primers at annealing temperatures of 50 and 55°C showed that some of the tested alpha streptococcal strains had $C_T$ values below 28, whereas *S. pneumoniae* at concentrations of $10^6$ had $C_T$ values around 26. At 60°C, *S. pneumoniae* had $C_T$ values around 16 at concentrations of $10^8$, whereas alpha streptococcal strains, at the same concentrations, had $C_T$ values over 30 or none at all. No $C_T$ values were obtained with the *Peptostreptococcus* spp. strains. Evaluation of specificity for the *frdB* primer showed low $C_T$ values at 50°C annealing temperature for some of the *H. parainfluenzae* strains earlier mentioned in this text, but the observed melting temperatures were out of range. Primers specific for *copB* amplified only *M. catarrhalis* at 50°C annealing temperature.

3.3. Reproducibility

A slight interassay variation in $C_T$ values for the dilution series of the positive controls could be observed. Table 1 shows the relationship between different concentrations of
Table 2

<table>
<thead>
<tr>
<th>Bacteria and runs</th>
<th>Amount of bacteria (CFU/mL)</th>
<th>10^8</th>
<th>10^7</th>
<th>10^6</th>
<th>10^5</th>
<th>10^4</th>
<th>10^3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>16.5</td>
<td>16.4</td>
<td>(0.6)</td>
<td>17.6</td>
<td>(6.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20.6</td>
<td>20.7</td>
<td>(0.5)</td>
<td>21.4</td>
<td>(3.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>24.6</td>
<td>24.4</td>
<td>(0.8)</td>
<td>25.5</td>
<td>(3.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>27.5</td>
<td>27.7</td>
<td>(0.7)</td>
<td>29.1</td>
<td>(1.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>31.2</td>
<td>31.7</td>
<td>(1.6)</td>
<td>32.1</td>
<td>(3.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. pneumoniae Run 1</td>
<td></td>
<td>14.9</td>
<td>13.5</td>
<td>(9.3)</td>
<td>13.7</td>
<td>(8.0)</td>
<td></td>
</tr>
<tr>
<td>S. pneumoniae Run 2</td>
<td></td>
<td>16.2</td>
<td>20.3</td>
<td>(6.8)</td>
<td>20.1</td>
<td>(5.7)</td>
<td></td>
</tr>
<tr>
<td>S. pneumoniae Run 3</td>
<td></td>
<td>19.0</td>
<td>24.9</td>
<td>(7.8)</td>
<td>24.7</td>
<td>(6.9)</td>
<td></td>
</tr>
<tr>
<td>H. influenzae Run 1</td>
<td></td>
<td>14.9</td>
<td>13.5</td>
<td>(9.3)</td>
<td>13.7</td>
<td>(8.0)</td>
<td></td>
</tr>
<tr>
<td>H. influenzae Run 2</td>
<td></td>
<td>16.2</td>
<td>20.3</td>
<td>(6.8)</td>
<td>20.1</td>
<td>(5.7)</td>
<td></td>
</tr>
<tr>
<td>H. influenzae Run 3</td>
<td></td>
<td>19.0</td>
<td>24.9</td>
<td>(7.8)</td>
<td>24.7</td>
<td>(6.9)</td>
<td></td>
</tr>
<tr>
<td>M. catarrhalis Run 1</td>
<td></td>
<td>8.9</td>
<td>9.0</td>
<td>(1.1)</td>
<td>9.7</td>
<td>(8.9)</td>
<td></td>
</tr>
<tr>
<td>M. catarrhalis Run 2</td>
<td></td>
<td>12.4</td>
<td>12.5</td>
<td>(1.0)</td>
<td>13.1</td>
<td>(3.5)</td>
<td></td>
</tr>
<tr>
<td>M. catarrhalis Run 3</td>
<td></td>
<td>20.3</td>
<td>20.4</td>
<td>(1.1)</td>
<td>21.0</td>
<td>(2.1)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3

<table>
<thead>
<tr>
<th>Comparison of significant pathogen findings with culture (≥10^5 CFU/mL) and RQ-PCR (corresponding to ≥10^4 CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RQ-PCR findings</td>
</tr>
<tr>
<td>S. pneumoniae</td>
</tr>
<tr>
<td>Run 1</td>
</tr>
<tr>
<td>12/203</td>
</tr>
</tbody>
</table>
| (5.9)                          | (5.4)
| H. influenzae                  | H. influenzae                  |
| –                              | –                              |
| –                              | –                              |
| –                              | (7.9)                          |
| M. catarrhalis                 | M. catarrhalis                 |
| –                              | –                              |
| –                              | 8/203                          |
| None                           | None                           |
| 2/203                          | 6/203                          |
| (1.0)                          | (3.9)                          |
| (0.5)                          | (69.5)                         |

Percent of all samples enclosed in parentheses.

* Positive in the atpA assay. Inhibition not present.

† Positive in the 16S rRNA assay, 13/14.

§ Positive in the 16S rRNA assay, 5/5. Inhibition present in 2/5 samples.

Table 4

<table>
<thead>
<tr>
<th>Cases with LRTI</th>
<th>RQ-PCR findings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. pneumoniae</td>
</tr>
<tr>
<td></td>
<td>H. influenzae</td>
</tr>
<tr>
<td></td>
<td>M. catarrhalis</td>
</tr>
<tr>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>5/135</td>
<td>10/135</td>
</tr>
<tr>
<td>(6.7)</td>
<td>(7.4)†</td>
</tr>
<tr>
<td>14/135</td>
<td>14/135</td>
</tr>
<tr>
<td>(11.9)</td>
<td>(10.4)§</td>
</tr>
<tr>
<td>8/135</td>
<td>1/135</td>
</tr>
<tr>
<td>(5.9)</td>
<td>(0.7)</td>
</tr>
<tr>
<td>5/135</td>
<td>75/135</td>
</tr>
<tr>
<td>(3.7)§</td>
<td>(55.6)§</td>
</tr>
</tbody>
</table>

Comparison of significant pathogen findings with culture (≥10^5 CFU/mL) and RQ-PCR (corresponding to ≥10^4 CFU/mL). Percent of all samples enclosed in parentheses.

* Positive in the atpA assay. Inhibition not present.

† Positive in the 16S rRNA assay, 13/14.

§ Positive in the 16S rRNA assay, 5/5. Inhibition present in 2/5 samples.

the same DNA run 3 times on different occasions. The intraassay variation was very low, as determined by running duplicates of the same samples in the same run (data not shown). Also, the C_T values obtained with different concentrations showed that 3–4 cycles corresponded to approximately one 10-log dilution, a pattern that was reproducible (Table 2).

3.4. Inhibition control

The positive controls all contained an amount of DNA corresponding to 25 000 CFU/mL. The ply, frdB, and copB assays produced average C_T values (SD) for the positive controls of respectively 29.51 (±0.43), 25.48 (±0.39), and 22.61 (±0.47). In the ply assay, 3/15 sputum samples and 1/15 invasive sample had C_T values above 2.5 SD of the positive controls, indicating partial inhibition. Inhibition was not detected in the 2 samples with significant cultures and insignificant ply RQ-PCR. Six samples contained significant amounts of H. influenzae judged by culture and insignificant amounts in the frdB RQ-PCR assay. Two of these samples, both containing >10^6 CFU/mL of H. influenzae, produced no C_T values, indicating complete inhibition. One sample had significant amount of M. catarrhalis according to culture and was insignificant in RQ-PCR; the inhibition control was negative in this sample.

3.5. Comparison of findings with culture and RQ-PCR

A higher number of pathogens was found with the RQ-PCR assay compared to the quantitative respiratory culture. Significant pathogen findings, defined as ≥10^5 CFU/mL, are summarized in Table 3. Significant pathogens were found in 45/203 (22.2%) samples with culture and in 68/203 (33.5%) with RQ-PCR (P < .05), corresponding to a 51% relative increase and an absolute increase in pathogen finding of 23/203 (11.3%) with RQ-PCR. The largest increase in the detection of a significant pathogen was seen for H. influenzae, where an absolute increase of 13/203 (6.4%) was observed with RQ-PCR.

Significant pathogen findings in relation to clinical signs of infection were also studied (Table 4). The total number of patients with clinical signs of lower respiratory tract infection (LRTI) was 135/189 (71.4%). The number of positive findings in this group of patients was 59/135 (43.7%). Fifty-one of these samples were found to be positive in the RQ-PCR, whereas 32 samples were positive according to culture, thus, resulting in an increase from 23.7% to 37.7% (P < .05). Significant RQ-PCR and insignificant culture was found in 10 samples with S. pneumoniae, 14 samples with H. influenzae, and 1 sample with M. catarrhalis. Antibiotic treatment before sampling was administered in 5/10, 3/14, and 0/1 of the respective samples. Almost all (8/9) cases of insignificant RQ-PCR
and significant culture were found in the group of patients with clinical signs of LRTI: 2 cases of *S. pneumoniae*, 5 cases of *H. influenzae*, and 1 case of *M. catarrhalis*. Antibiotic treatment before sampling in this group was only given in 2 cases, both with *H. influenzae*.

In the group of patients with no overt signs of LRTI, 9/54 samples (16.7%) had significant amounts of 1 of the examined pathogens. *S. pneumoniae* was found in 3 cases with both methods. Two of these patients suffered from lung cancer, and 1 patient had asthma bronchiale. Significant amount of *H. influenzae* was found in 5 cases. In 2 of the cases (both with cardiac events, 1 treated with antibiotics before sampling), the pathogen was only found with RQ-PCR; in 1 case (asthma bronchiale), only with culture; and in 2 cases, with both methods (COPD). *M. catarrhalis* was found in 1 sample with RQ-PCR only, in a patient suffering from bronchiectasis.

Chronic obstructive pulmonary disease or other chronic pulmonary disease was present in 70/190 patients, out of which 46 patients were considered to have LRTI. Among the patients with no apparent signs of LRTI, *S. pneumoniae* was found in significant amount in 3 samples, *H. influenzae* in 2 samples, and *M. catarrhalis* in 1 sample. *S. pneumoniae* was found with both culture and RQ-PCR, whereas *M. catarrhalis* and 1 case of *H. influenzae* were detected only by RQ-PCR. Discrepancies between culture and RQ-PCR were found in 10 samples. Significant RQ-PCR and insignificant culture were found in 3 cases for *S. pneumoniae* and 4 cases for *H. influenzae*, whereas significant culture and insignificant RQ-PCR were found in 3 cases for *H. influenzae*.

Further analysis of the group of samples with significant pathogen findings in RQ-PCR and insignificant or negative culture (n = 28) showed that the majority was culture negative. Some of the samples had such discrepancies for more than 1 of the tested pathogens. When sorting by pathogens, the total number of discrepancies between culture and RQ-PCR was 32, and only 7/32 (21.9%) samples had detectable amounts of the respective pathogens in culture, whereas the rest of the samples were culture negative or reported as regular respiratory flora. All 11 samples with negative or insignificant culture and significant ply RQ-PCR were also positive in the *atpA* assay. For *H. influenzae*, 15/19 samples that were significant in the *frdB* assay were positive in the qualitative PCR targeting the *16S rRNA* gene, whereas the remaining 4 were negative or presented unspecific bands.

Significant culture and insignificant or negative RQ-PCR were found for *S. pneumoniae* in 2/203 samples (0.9%), for *H. influenzae* in 6/203 samples (3.0%), and for *M. catarrhalis* in 1/106 (0.9%) (Table 3). The 2 samples that were culture positive for *S. pneumoniae* had no detectable DNA in the ply RQ-PCR assay but were found to be positive in the *atpA* assay. Regarding *H. influenzae*, 1 sample with significant culture had no detectable DNA in the *frdB* assay, whereas the other 5 samples had levels of DNA corresponding to 10³–10⁴ CFU/mL. Inhibition was present in 2/6 samples that had ≥10⁵ CFU/mL *H. influenzae* according to culture, but not in the last 4. Furthermore, all samples were PCR positive with the 16S rRNA assay, although the 2 samples featuring inhibition displayed weak bands. The only sample with significant culture and insignificant RQ-PCR of *M. catarrhalis* contained an amount of DNA corresponding to 10⁴ CFU/mL.

Insignificant culture and RQ-PCR findings are shown in Tables 5–7. For *S. pneumoniae*, this combination was found in 178/203 cases, for *H. influenzae* in 162/203 cases, and for *M. catarrhalis* in 192/203 cases. Negative culture in combination with RQ-PCR findings corresponding to 10⁴ CFU/mL was observed in 15 cases, 12 of them with
H. influenzae. Negative RQ-PCR and culture finding of $10^5$ CFU/mL was seen in 4 cases.

4. Discussion

In this material consisting of samples from patients with various clinical conditions, RQ-PCR substantially improved significant pathogen detection. Conflicting evidence exist regarding the impact of bacteriologic investigations on therapeutic outcome in pneumonia. In the case of nonsevere pneumonia, several authors have come to the conclusion that no beneficial effect is detectable (Levy et al., 1988; Sanyal et al., 1999; Theerthakarai et al., 2001). A common trait for these studies has been the low rate of identification of causative agents, which could possibly contribute to the observed lack of impact on therapeutic outcome. In studies of severe pneumonia where detection rates generally have been higher, microbiologic investigations have been regarded important (Örtqvist, 1994; Rello et al., 2003).

Also, in all cases of pneumonia, an etiologic diagnosis has the potential of permitting pathogen-directed antimicrobial chemotherapy with less need for the use of more resistance-driving broad-spectrum antibiotics. In the current study, we found that among the patients with clinical signs of LRTI, significant amounts of S. pneumoniae, H. influenzae, and M. catarrhalis were present in 44% of the samples when combining culture and RQ-PCR. Conversely, significant amounts of the 3 pathogens were only present in 16% in the group of patients without overt signs of LRTI, and also 8/9 patients in this group had COPD or other chronic pulmonary disease. The proportion of positive samples in the LRTI group was remarkably high, considering the heterogeneous material and the fact that we did not examine the role of other common pathogens, such as M. pneumoniae. Thus, we believe that future studies on the importance of microbiologic investigations for the clinical outcome of LRTI should apply more sensitive detection methods.

The great increase in significant pathogen detection observed with RQ-PCR could theoretically be explained by false-positive samples, although the primers used for detection appeared species specific. A general problem in validation of RQ-PCR–positive samples is the lack of an appropriate golden standard because both respiratory cultures and blood cultures have low sensitivities for the detection of respiratory pathogens. To support our findings, we therefore chose to enhance species identification by including additional species-specific primers. For S. pneumoniae, atpA primers generated specific PCR products in all samples that were culture negative and significant according to RQ-PCR, supporting the correctness of our initial findings. For H. influenzae, results by use of 16S rRNA primers supported our findings for 15/19 samples with negative cultures and significant findings in the frdB assay. The remaining 4 samples became negative with the 16S rRNA assay, raising the possibility that they could be false positives. For M. catarrhalis, negative culture and significant RQ-PCR were observed in only 2 samples. Primers targeting the copB gene have earlier been reported to have excellent specificity for M. catarrhalis (Greiner et al., 2003). Also, both of these samples were reported as having overgrowth of other bacteria, which might have had a negative impact on culture identification.

Several factors may contribute to the observed differences in the rate of significant pathogen detection between respiratory cultures and RQ-PCR. The sensitivity of quantitative respiratory cultures may be negatively influenced by the presence of much respiratory commensals, as well as the antibiotic treatment before sampling. The presence of commensals may impair both identification and quantification of potential pathogens, leading to false-negative cultures or quantitative underestimation of relevant pathogens. Earlier studies have also demonstrated that respiratory samples may become negative after a short course of antibiotic treatment (Elmes et al., 1953; Kalin and Lindberg, 1983a; Garcia-Vazquez et al., 2004; Musher et al., 2004), and that PCR might remain positive in such samples (Wheeler et al., 1999). In this study, we found that in 8/25 cases (32%) where such discrepancies were observed, antibiotic treatment with drugs active against the causative agents had been given before sampling, theoretically explaining one-third of the additional cases found with RQ-PCR. Lastly, optochin resistance in S. pneumoniae may also lead to misclassification because this is the main phenotypical method for species identification (Munoz et al., 1990).

Significant culture and insignificant RQ-PCR for the 3 examined pathogens was observed in 9 samples, 8 of them being sputum samples. In 2 samples containing $10^5$ CFU/mL of S. pneumoniae according to culture, RQ-PCR was negative. The inhibition assay indicated that inhibition was not present in these 2 samples. The samples were both positive with the atpA assay, which is the genotypic correlate to optochin susceptibility. Optochin-susceptible S. mitis has been described (Martín-Galiano et al., 2003) and could theoretically have been present in these samples. Alternatively, presence of S. pneumoniae with altered pneumolysin sequence could result in negative PCR.

In samples with significant concentration of H. influenzae as estimated by culture and insignificant or negative PCR, 2 of 6 samples showed evidence of inhibition, which might explain that frdB PCR was negative or insignificant in these samples. In 2 of the remaining 4 samples, the amount detected with culture was $10^6$ CFU/mL, whereas PCR detected $10^4$ CFU/mL. In the last 2 samples, $10^5$ and $10^6$ CFU/mL were detected with culture, whereas the amount of DNA detected with PCR corresponded to less than $10^4$ CFU/mL. All 6 samples were positive with the 16S rRNA assay, but because this is a qualitative assay, it is impossible to elucidate the question of quantity. One possible explanation could be mutations in the target gene, leading to imperfect hybridization of the primers and
consequently impaired amplification. For *M. catarrhalis*, 1 sample contained 10⁶ CFU/mL according to culture, and only 10⁴ CFU/mL according to PCR. Because no inhibition was present in this sample, the possibility of presence of target gene mutations could also be raised in this case.

Summarized, the RQ-PCR assay substantially increased the number of significant findings of the 3 examined pathogens. Because the assay is quantitative, positive findings reflecting a high concentration of bacteria probably represent clinically significant infection with the respective pathogen, supported also by the clinical data presented in this study. Another important feature of the assay is the usefulness on all categories of lower respiratory tract specimens. Furthermore, the method is simple, rapid, and seems to be useful particularly in cases where short antibiotic treatment has been administered before sampling.

The cost of an RQ-PCR analysis will largely depend on local laboratory factors and may also decrease over time. At present, the cost of an RQ-PCR analysis at Karolinska University Hospital is around US$100, whereas the cost of a respiratory culture is approximately US$50. These sums may be compared with the cost of 1 day of hospital care, which is approximately US$600. Because respiratory cultures are of importance for both susceptibility testing and detection of a broader range of bacteria, combination with culture may be the best way to use the potential of RQ-PCR. The use of both methods would theoretically increase cost per sample 3-fold, but could probably still be motivated by the potential of increasing pathogen finding, as well as decreasing time consumption to microbiologic diagnosis by at least 24 h. Studies on the impact of microbiologic findings obtained with novel diagnostic protocols on clinical outcome in LRTI are warranted because such data will largely decide whether the increased cost could be motivated.

Acknowledgments

The authors thank Martin Vondracek, Kristina Fahlander, Hector Roldan, Siri Wahlquist, Inga Karlsson, and Carina Bengtsson for excellent technical assistance.

We would also like to thank Professor Richard Moxon, Oxford, for technical advice.

References


