Application of quantitative PCR to the diagnosis and monitoring of *Pseudomonas aeruginosa* colonization in 5–18-year-old cystic fibrosis patients

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Received 30 June 2010
Accepted 13 October 2010

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Early detection of *Pseudomonas aeruginosa* and early aggressive treatment are recommended to delay chronic infection in cystic fibrosis (CF) patients. The aim of this study was to assess a quantitative PCR (q-PCR) assay for the diagnosis of early *P. aeruginosa* colonization in 23 young CF patients (group A, age range 7–18 years) and to survey the eradication of *P. aeruginosa* in 10 young CF patients (group B, age range 5–18 years) after an initial antibiotic treatment. q-PCR results for consecutive sputum samples from each patient during a period of 18 months were compared with bacterial cultures during the same period plus an additional period of 12 months, and with concomitant clinical signs of pulmonary exacerbation. The q-PCR and bacterial cultures were negative for 17 of the 23 patients in group A and six of the 10 patients in group B during the study period. However, consecutive positive q-PCR results were observed for one patient in group A and three patients in group B, while the bacterial cultures for the same sputum sample remained negative. They preceded positive *P. aeruginosa* bacterial cultures at 7 and 8 months for two patients in group B. These positive results were associated with a worsening of the clinical status of patients, but pulmonary exacerbation appeared non-specific for the diagnosis of early *P. aeruginosa* colonization since pulmonary exacerbations were observed in patients in whom q-PCR or bacterial culture remained negative. In conclusion, q-PCR may be a useful additional tool to provide information on the *P. aeruginosa* status of CF patients.

INTRODUCTION

*Pseudomonas aeruginosa* is the most important cause of lung infections in patients with cystic fibrosis (CF). It is often acquired in the first years of life and causes chronic airway infections that lead to respiratory failure and ultimately result in mortality. Aggressive antibiotic therapy is the most efficient means to eradicate early *P. aeruginosa* colonization and to postpone the chronic infective stage (Frederiksen et al., 1997, 1999; Høiby et al., 2005; van Belkum et al., 2000).

Detection of *P. aeruginosa* is usually achieved by culturing homogenized sputa or oropharyngeal swabs onto specific and selective media. However, bacterial culture has limited sensitivity for detecting very low numbers of bacterial cells (De Vos et al., 2001; van Belkum et al., 2000). The two critical situations are the diagnosis of early colonization and the total eradication of these early colonizations after antibiotic treatment.

To improve early detection of *P. aeruginosa* colonization, several molecular assays have been developed (Karpati & Jonasson, 1996; van Belkum et al., 2000; Xu et al., 2004). Xu et al. (2004) demonstrated that conventional PCR based on the amplification of the oprL (outer-membrane lipoprotein) and exoA (exotoxin A) genes directly in sputum samples of CF patients allowed the detection of new colonizations. However, sensitivity was not optimal and 8.8% of CF patients were culture-positive and PCR-negative for both gene targets. Quantitative PCR is a reliable and more sensitive method (Pirnay et al., 2000), but there is a lack of information about its use directly on clinical samples.

Abbreviations: CF, cystic fibrosis; Cq, cycle of quantification; q-PCR, quantitative PCR.
The aim of this study was to assess a quantitative PCR (q-PCR) assay for the diagnosis of early \( P. \) aeruginosa colonization and to survey \( P. \) aeruginosa eradication or persistence after early and aggressive antibiotic treatment in 5–18-year-old CF patients. q-PCR results of consecutive sputum samples collected from each patient during a period of 18 months were compared with bacterial cultures during the same period plus an additional period of 12 months, and with concomitant clinical signs of pulmonary exacerbation.

**METHODS**

**Patients.** Thirty-three CF children (mean age: 12 years; range: 5–18 years) attending the Cystic Fibrosis Center of Jeanne de Flandre Lille University Children’s Hospital were involved in this study. Patients were divided into two groups according to their past history of \( P. \) aeruginosa colonization evaluated from bacterial culture of their sputum. Characteristics of the two target populations are summarized in Table 1. Group A included 23 patients (age range 7–18 years) who had never been colonized by \( P. \) aeruginosa and group B (age range 5–18 years) included 10 in whom \( P. \) aeruginosa had been detected at least once in the prior 3 years and who had received specific aggressive anti-\( \text{Pseudomonas} \) treatment (ceftazidime–tobramycin combination for 2 weeks or ceftazidime–ciprofloxacin combination for 3 weeks, both combinations followed by aerosolized tobramycin for at least 1 month). The time between the last positive culture of \( P. \) aeruginosa and the first analysis for the study varied from 4 to 32 months.

Sputum samples were collected in sterile plastic containers during routine outpatient visits, either spontaneously or after chest physiotherapy, over a period of 18 months. During this period, a total of 72 and 31 sputa were collected from patients in group A and B, respectively, with a mean of three sputa per patient (range two to five samples per patient).

**Pulmonary exacerbation.** For all patients, presence of pulmonary exacerbation was investigated using the score of Rosenfeld (Rosenfeld et al., 2001a) at the time of sample collection. This score is based on functional signs that are matched as follows: increased cough (1.5 points), increased sputum/cough congestion (1.5 points), school absenteeism (1.6 points), increased adventitial sounds on lung examination (1.2 points), decreased appetite (1.1 points) and decreased exercise tolerance (1.8 points). Pulmonary exacerbation was defined by a final score equal to or superior to 2.6 after adding the points obtained for each item.

<table>
<thead>
<tr>
<th>Table 1. Characteristics of group A and group B CF patients</th>
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<tbody>
<tr>
<td><strong>Group A</strong></td>
</tr>
<tr>
<td><strong>(n=23)</strong></td>
</tr>
<tr>
<td><strong>Mean age, years (range)</strong></td>
</tr>
<tr>
<td><strong>Sex ratio, M/F</strong></td>
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<tr>
<td><strong>Colonized by ( H. ) influenzae</strong></td>
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<tr>
<td><strong>Colonized by ( Staphylococcus aureus )</strong></td>
</tr>
<tr>
<td><strong>Colonized by ( Stenotrophomonas maltophilia )</strong></td>
</tr>
<tr>
<td><strong>Colonized by ( P. ) aeruginosa</strong></td>
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</table>

*Time between the last positive culture of \( P. \) aeruginosa and the study varied from 4 to 32 months.

**Culture techniques.** Sputum cultures were collected during a total period of 30 months. Part of the sputum sample was mixed with an equal amount of sputolysin. Aliquots (100 μl) were uniformly plated out onto specific agar plates (bioMérieux) for the detection of \( P. \) aeruginosa (cetrimide agar) and Burkholderia cepacia complex strains (\( B. \) cepacia agar) and three other media (IsoVitalex chocolate Columbia agar with 5% sheep blood, Columbia agar with 5% sheep blood with nalidixic acid and Bromocresol Purple agar) for the other pathogens usually found in CF patients. The plates were incubated aerobically at 37 °C (30 °C for \( B. \) cepacia agar) and inspected for \( P. \) aeruginosa colonies after 24–72 h. \( P. \) aeruginosa strains were identified according to standard methods using the oxidase test and API 20N (bioMérieux).

**Preparation of bacterial DNA from sputum.** One millilitre of liquefied (dithiothreitol-treated) sputum was centrifuged at 11 000 g for 15 min. Aliquots of 0.1 ml of the pellet were used in order to obtain a 10-fold concentration of the samples. Bacterial DNA was extracted using the RTP Spin DNA Mini kit (Invitex Laboratory Eurobio) and stored at −20 °C before amplification by q-PCR. In each experiment, a negative control composed of all reagents except sputum and a positive control corresponding to sputum collected from a chronic \( P. \) aeruginosa-colonized patient were included.

**\( P. \) aeruginosa detection by real-time PCR.** q-PCR targeting the oprl gene was performed in the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Sequences of primers and probe were: forward primer, 5’-AACAGGCGTGCGGTGAC-3’; reverse primer, 5’-GTCGGAGCTGTCTGACTGAA-3’; and DNA probe, 6-FAM TGAGCGAGAAGCC MGB (Miner Groove Binder). Assays were performed in 25 μl containing 5 μl DNA template and 200 nM each primer and probe, in a 1× TaqMan Universal PCR Master mix. Thermocycling conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 45 cycles at 95 °C for 15 s and 60 °C for 1 min. Fluorescence was measured after each cycle. Each assay was carried out in duplicate and the median Cq (cycle of quantification) value from each duplicate was used for analysis. The specificity of the assay was assessed against 61 bacteria usually found in the sputum of CF patients: \( Stenotrophomonas maltophilia \) (n=17), \( Acinetobacter xylosoxidans \) (n=12), \( B. \) cepacia complex (n=2), \( Haemophilus influenzae \) (n=2), \( Haemophilus parainfluenzae \) (n=1), \( Pseudomonas \) species (n=6), enterobacteria (n=5), \( Staphylococcus aureus \) (n=2), atypical mycobacteria (n=5), \( Streptococcus \) species (n=8) and \( Acinetobacter baumannii \) (n=1).

**Quantification.** To quantify the number of bacterial cells in sputum, serial 10-fold dilutions were performed from a \( P. \) aeruginosa PAO-1 culture grown to the mid-exponential phase. To correlate the value of Cq measured by q-PCR with the potential number of \( P. \) aeruginosa cells present in each sample, aliquots (100 μl) of each dilution of bacterial suspension were plated out in triplicate onto Bromocresol Purple agar (bioMérieux). Agar plates were incubated overnight at 37 °C, and colonies were counted in order to calculate the number of c.f.u. per dilution tube. A sample (5 μl) from each dilution tube was extracted with the same protocol as DNA extraction from sputum and was analysed concomitantly by q-PCR. The number of micro-organisms in sputum samples was determined by comparing Cq values.

**Statistical analysis.** Groups were compared using the Fisher’s exact test. Differences were considered statistically significant for \( P<0.05 \).

**RESULTS**

**Detection limit and specificity of the q-PCR assay**

The q-PCR assay performed on a serial 10-fold dilution of \( P. \) aeruginosa showed a linear curve for quantification with
a detection limit of 150 c.f.u. ml$^{-1}$. By taking into account the 10-fold concentration of our sputum by centrifugation, we could conclude that the detection limit of the q-PCR assay in sputum corresponded to 15 bacteria ml$^{-1}$. The oprL gene was highly specific for P. aeruginosa. The q-PCR tested against a panel of 61 strains was only positive with P. aeruginosa strains.

Detection and quantification of P. aeruginosa by q-PCR

q-PCR was performed on each sputum sample collected from the 23 patients in group A (72 samples) and the 10 patients in group B (31 samples) over a period of 18 months. Although sputum culture did not allow the detection of P. aeruginosa strains during this study, P. aeruginosa DNA was detected by q-PCR in seven samples from patients in group A and 12 samples from patients in group B. By comparing the results with the standard curve from P. aeruginosa culture, the median number of P. aeruginosa genome equivalents (ml sputum)$^{-1}$ corresponded to 28 (range 16–79) in group A. It was significantly higher in group B with a median number of P. aeruginosa genome equivalents ml$^{-1}$ of 790 (range 63–1540).

Association between P. aeruginosa detection and pulmonary exacerbation

Clinical signs of pulmonary exacerbation were measured during each sample collection (Table 2). Negative q-PCR results were more often observed when the clinical status was stable than during pulmonary exacerbation, but this association was not statistically significant in either group. Positive q-PCR results coincided with a clinical decline in three cases for the patients in group A and in seven cases for the patients in group B, and were observed in four cases for the patients in group A and in five cases for the patients in group B during the clinically stable period.

Follow-up of q-PCR results over time

The follow-up of q-PCR results was analysed for each patient over 18 months and compared with the bacterial culture during the same period, plus an additional period of 12 months (Table 3). q-PCR assay and culture remained negative for 17 patients in group A and six patients in group B. q-PCR results varied over time for five patients in group A and one in group B, but the bacterial culture remained negative throughout the study. Consecutive positive q-PCR results were observed for one patient in group A and three patients in group B. The bacterial culture remained negative over time for the patient in group A and for one of the patients in group B, whereas P. aeruginosa was detected by culture 7–8 months later (mean 7.5 months) for the two other patients in group B. Comparison of these positive q-PCR results with clinical status showed that these two patients in group B suffered pulmonary exacerbation at the sampling time.

DISCUSSION

In agreement with published data (Deschaght et al., 2009; De Vos et al., 1997; Pirnay et al., 2000; Xu et al., 2004), we demonstrated that the q-PCR method using the oprL gene was highly specific and more suitable than culture for detecting P. aeruginosa directly in CF sputum. The limit of detection of 15 bacteria ml$^{-1}$ was excellent. Using our highly sensitive q-PCR technique, we found that P. aeruginosa DNA was detected in the sputum of 6/23 patients in group A and 4/10 in group B, whereas P. aeruginosa was not detected using classical culture procedures.

However, by following q-PCR results over 18 months and by comparing the results with bacterial culture during the

Table 2. Comparison of q-PCR results with clinical score of Rosenfeld (Rosenfeld et al., 2001a) concomitantly measured during each sample collection

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<tr>
<th></th>
<th>Group A</th>
<th>Group B</th>
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<tbody>
<tr>
<td></td>
<td>Score ≤2.6</td>
<td>Score ≥2.6</td>
</tr>
<tr>
<td>q-PCR −</td>
<td>55</td>
<td>10</td>
</tr>
<tr>
<td>q-PCR +</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
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Table 3. Follow-up of q-PCR results for 18 months and bacterial culture for 30 months

<table>
<thead>
<tr>
<th>q-PCR (18 months)</th>
<th>Culture (30 months)</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Always negative</td>
<td>Always negative</td>
<td>17</td>
<td>6</td>
</tr>
<tr>
<td>Varied q-PCR results*</td>
<td>Becoming positive</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Always positive</td>
<td>Always negative</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Becoming positive</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Negative results followed by a positive result were observed for two patients in group A and one patient in group B; positive result followed by negative results were observed for three other patients in group A.
same time plus an additional period of 12 months, we showed that some positive q-PCR results were observed in only one sample of the two, three or four collected. This situation was observed especially for patients in group A who had never been infected before the study and correlated with very low numbers of P. aeruginosa genome equivalents of between 16 and 79 ml⁻¹. These positive q-PCR results could not be considered a false-positive relative to possible contamination in our method, because negative controls were included and all precautions were taken for each PCR assay. These isolated positive q-PCR results suggested either a very intermittent and low colonization of the airways or an oropharyngeal contamination of sputum by P. aeruginosa DNA, which might result from dead or non-viable bacteria.

Consecutive positive q-PCR results were observed for one patient in group A and three patients in group B, while the bacterial culture of all their sputum samples remained negative for P. aeruginosa. As above, these positive q-PCR results might mean the presence of DNA from non-viable or dead bacteria. Since P. aeruginosa was still detected in patients in group B before the study, the persistence of DNA might occur at the surface of the mucous membrane. After examining the medical records of these patients, the last detection of P. aeruginosa by culture ranged from 4 to 9 months. In the future, studies targeting specific P. aeruginosa mRNA and clonality might be an efficient tool to define viable but non-cultivable bacteria and new or persistent colonization. Interestingly, the detection of P. aeruginosa by culture of sputum for two of the three patients in group B after 7 and 8 months suggested the beginning of a new P. aeruginosa infection that could only be detected by q-PCR. The number of bacteria estimated by comparing the results of q-PCR with the standard curve of P. aeruginosa cells was significantly higher for these two cases than in previous patients (mean of 930 P. aeruginosa genome equivalents ml⁻¹ and 1330 P. aeruginosa genome equivalents ml⁻¹, respectively) and these positive q-PCR results were associated with a worsening of their clinical status. However, the comparison of clinical signs defining pulmonary exacerbation to q-PCR and culture at each sampling time was not very informative for the other patients. These signs were also observed in patients in whom P. aeruginosa q-PCR or bacterial culture remained negative. This result complemented other studies demonstrating the absence of clinical symptoms during early P. aeruginosa colonization of airways (Burns et al., 2001; Rosenfeld et al., 2001b, 2003). Conversely, the worsening of pulmonary symptoms was observed in some patients while no P. aeruginosa DNA was detected by q-PCR. This finding was attributed to the presence of other bacteria such as Staphylococcus aureus, which was always present in their sputum.

In conclusion, due to the sensitivity and specificity, our q-PCR assay allows the detection of very low numbers of P. aeruginosa. This method may be a very useful tool in association with bacterial culture for the diagnosis of early P. aeruginosa colonization and the survey of P. aeruginosa eradication after antimicrobial therapy in young CF patients.

ACKNOWLEDGEMENTS

We thank the nursing and laboratory staff for their helpful assistance.

REFERENCES


