Comparative Evaluation of Molecular Detection Performance of Pseudomonas aeruginosa based on Phylogenetic Markers 16S RNAr, recA, rpoB and ITS1

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Introduction

Pseudomonas aeruginosa is a ubiquitous bacterium found in the environment such as soil, water, inert surfaces, plants, antiseptic solutions and foods [1-3]. It is also a commensal of the digestive tract that can reach high populations and can cause infections in immunocompromised persons and immunocompromised subjects or weakened, causing a high rate of morbidity and mortality. The aim of this study was to determine the phylogenetic marker suitable for molecular identification of Pseudomonas aeruginosa. The purity and concentration of the nucleic acids were determined by spectrophotometry. Sensitivity reactions using phylogenetic markers (16S RNAr, recA, rpoB, STS1) and the threshold detection of 42 strains were assessed by polymerase chain reaction (PCR). With an average absorption at 230 nm of 2.1, the DNA extracts has an average ratio (A260/A280) of 1.7. The threshold detection of Pseudomonas aeruginosa reference strain ATCC 27853 was 0.8 µg/ml for rpoB and 7.6 µg/ml for each of 16S markers RNAr and recA. The threshold detection of positive control strains CP2: 1125A and CP3: API was 1.2 µg/ml and 0.1 µg/ml for using rpoB gene, respectively. This threshold was respectively 12.3 µg/ml and 0.9 µg/ml for the recA gene. The sensitivity of the rpoB housekeeping gene was 97.4% followed by the recA and 16S RNAr with 87.2% and 82.1%, respectively. The phylogenetic resolution of the rpoB genes was higher than that of the 16S rRNA and recA genes. No sensitivity reaction was observed with ITS1 marker. The quality, purity of the nucleic acids and the choice of phylogenetic marker are among the most critical factors for PCR analysis.

Material and Methods

Pseudomonas aeruginosa strains

Forty-two (42) strains of Pseudomonas aeruginosa, consisting of thirty-nine (39) strains isolated from animal products and three (3) positive control strains from Institut Pasteur of Côte d’Ivoire were used in this study. The 3 positive controls are Pseudomonas aeruginosa ATCC 27853, Pseudomonas aeruginosa 1125A and Pseudomonas aeruginosa API.
Sensitivity evaluation

In total, three (3) positive controls and thirty nine (39) presumptive isolates of *Pseudomonas aeruginosa* were used. Genomic DNA was extracted by boiling and purified according to the method previously described [18]. After culturing for 24 hours in 2 ml Brain Heart broth (Biokar Diagnostics, BK015HA, France) at 37°C, the strains were grown on Müeller Hinton agar (Biokar Diagnostics, BK048HA, France) for 18-24 h. Three (3) colonies were picked and suspended in 1 ml of Milli-Q water (milli-Q™, Millipore Corporation, USA) and 500 μl of absolute ethanol (Agilent Technologies, CA, USA). The mixture reaction to determine the threshold detection and sensitivity by PCR, targeting housekeeping genes RNAr16S, recA, rpoB and ITS1.

All the PCR were performed in a volume of 25 μl of bacterial genomic DNA solution consisted of 16 μl of sterile Milli-Q water (milli-Q™, Millipore Corporation, USA), 5 μl of loading buffer (5X) concentration, 1.5 μl of MgCl₂ (2 mM) (Promega Corporation, Madison, WI 53711-5399, USA), 0.2 μl dNTPs (10 mM), 0.1 μl of each primer (20 mM) (Integral DNA Technology, France), 0.1 μl of Go Tag G2 Flexi DNA polymerase final concentration 1.5 U (Promega Corporation, Madison, WI 53711-5399, USA) and 2 μl of the DNA template. Water sterile Milli-Q and the reference strains of *Pseudomonas aeruginosa* ATCC 27853 were used respectively as negative control and positive control for each PCR reaction.

**Amplification of 16S rRNA, recA, rpoB and ITS**

The amplification of 16S rRNA gene for *Pseudomonas* detection was performed according to the method described. The determination of *Pseudomonas aeruginosa* is carried out by amplification of specific fragments of recA, rpoB and ST11. The amplification of recA, rpoB and ITS genes has achieved respectively by using primer pair rec-AS and recA-AS; rpoB-F and rpoB-R and 16F945 and 23R458 as described [19-22]. The amplification program and the nucleotide sequence of the primers used are described in (Table 1). The amplification reactions were performed in a thermocycler type T3000 Thermocycler, Block standard type 3a (Biomat, Germany). 10 μl of PCR products were revealed on a 2% agarose gel for 30 min at 120 V. A volume of 5 μl of a molecular weight marker (Bench Top, 1kb DNA Ladder, Promega Corporation, USA) was included. The gels were prepared in 1X TAE buffer containing 2.5 mg/L of ethidium bromide solution and visualized by UV transilluminator (Molecular Imager Gel DocTM EZ, Bio-Rad, USA).

### DNA extraction

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**Mixed reaction of gene amplification**

After determining purity and concentration of DNA in the extract by spectrophotometry decimal dilutions ranging from 10⁻¹ to 10⁻¹⁰ were conducted to the DNA suspension constitution for each strain. The different DNA suspension lines were used as template DNA in the mixture reaction to determine the threshold detection and sensitivity by PCR, targeting housekeeping genes RNAr16S, recA, rpoB and ITS1.

### Sensitivity evaluation

The sensitivity of molecular test refers to the ability of the test to correctly identify *Pseudomonas aeruginosa* strains in samples with the phylogenetic marker. This test demonstrates the most sensitive and rapid method using phylogenetic markers for the detection of *Pseudomonas aeruginosa* strains. When evaluating a molecular test,
the expression for calculating sensitivity according to Ghaaliq and Mccluskey is as follows [21].

$$\text{Sensitivity} = \frac{\text{True Positives}}{\text{True Positives} + \text{False Negatives}}$$

In that expression, True positive show that *Pseudomonas aeruginosa* is present in the sample and the test is positive; while, false positive show that *Pseudomonas aeruginosa* is not present in the sample but the test is positive. In this study, all strains of *Pseudomonas aeruginosa* studied were isolated, identified and stored in the laboratory with well-known characteristics. The various molecular tests allowed for selecting the appropriate phylogenetic marker to confirm this identification.

**Statistical analysis**

Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) Version 22; SPSS Inc., Chicago, IL, USA. The P value of <0.05 was deemed as statistically significant.

**Results**

**Concentration and purity of the extracted nucleic acids**

The DNA extracts has an average ratio (A260/A280) of 1.7. This ratio is close to 1.8 and indicates the purity of DNA preparations concerning proteins, phenol or agarose. Mean absorption at 230 nm is 2.1 and also reflects the non-contamination of the preparations by substances such as carbohydrates, peptides or aromatic compounds (Table 2).

<table>
<thead>
<tr>
<th>No order</th>
<th>Sample Type</th>
<th>Purity A&lt;sub&gt;260&lt;/sub&gt;/A&lt;sub&gt;280&lt;/sub&gt;</th>
<th>Purity A&lt;sub&gt;260&lt;/sub&gt;/A&lt;sub&gt;230&lt;/sub&gt;</th>
<th>Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CP&lt;sub&gt;1&lt;/sub&gt;: ATCC 27853</td>
<td>1.7</td>
<td>2.1</td>
<td>75.5</td>
</tr>
<tr>
<td>2</td>
<td>CP&lt;sub&gt;2&lt;/sub&gt;: 1125A</td>
<td>1.7</td>
<td>2.2</td>
<td>122.7</td>
</tr>
<tr>
<td>3</td>
<td>CP&lt;sub&gt;3&lt;/sub&gt;: API</td>
<td>1.7</td>
<td>2.1</td>
<td>88.5</td>
</tr>
</tbody>
</table>

**Table 2**: Values of absorbance and concentration of DNA extracts from positive controls (CP: positive control). 

**Detection of *Pseudomonas aeruginosa* strains by phylogenetic marker**

The detection limits were evaluated with the three positive controls strains from the genes of households 16S RNAr, recA, rpoB and STI1. With an initial DNA concentration of 75.5 µg/ml and a purity of 1.7 for *Pseudomonas aeruginosa* ATCC 27853 reference strain, good resolution is achieved only with the rpoB gene for a detection limit to 0.8 µg/ml (Table 2 and 3). *Pseudomonas aeruginosa* ATCC 27853 reference strain threshold detection by each of 16S markers RNAr and recA was 7.6 µg/ml (Figures 1-3). The best threshold detection of positive controls strains CP<sub>2</sub>: 1125A and CP<sub>3</sub>: API was respectively 1.2 µg/ml and 0.1 µg/ml with rpoB marker. The threshold detection with the recA gene was 12.3 µg/ml for strain CP<sub>2</sub>: 1125A (Figures 4-8) and 0.9 µg/ml for strain CP<sub>3</sub>: API. These thresholds detection of strains CP<sub>2</sub>: 1125A and CP<sub>3</sub>: API has been obtained with DNA initial concentrations respectively of 122.7 µg/ml and 88.5 µg/ml for a purity of approximately 1.7 (Tables 2 and 3; Figure 9-12). No threshold detection was observed with ITS1 gene (Figure 4).
Figure 4: Electrophoretic profile showing the threshold detection of the ITS gene from *P. aeruginosa* ATCC 27853 (M: Marker Gene Ruler 250 bp).

Figure 5: Electrophoretic profile showing the threshold detection of the 16S RNA gene from *P. aeruginosa* 1125A (M: Marker Gene Ruler 250 bp).

Figure 6: Electrophoretic profile showing the threshold detection of the *rpoB* gene from *P. aeruginosa* 1125A. (M: Marker Gene Ruler 250 bp).

Figure 7: Electrophoretic profile showing the threshold detection of the *recA* gene from *P. aeruginosa* 1125A. (M: Marker Gene Ruler 250 bp).

Figure 8: Electrophoretic profile showing the threshold detection of the ITS gene from *P. aeruginosa* 1125A (M: Marker Gene Ruler 250 bp).

Figure 9: Electrophoretic profile showing the threshold detection of the 16S RNA gene from *P. aeruginosa* API (M: Marker Gene Ruler 250 bp).
The sensitivity of the reaction with using four markers phylogenetic 16S RNAr, recA, rpoB and ITS1 for Pseudomonas aeruginosa identification indicates that a sensitivity of the rpoB housekeeping gene is 97.4% followed by the recA and 16S RNAr respectively 87.2% and 82.1% (Table 4) (Figures 13 to 15). However, no sensitivity reaction was observed with using ITS1 marker (Table 4 and Figure 16).

Table 3: Threshold detection of Pseudomonas aeruginosa strains by phylogenetic markers. Each index letter indicates threshold detection values for the various phylogenetic markers whose column proportions do not differ significantly from each other in level 05. The Threshold detection indicates the lowest concentration of DNA from which each phylogenetic marker can always detect the strains of Pseudomonas aeruginosa with a known initial concentration (CP: positive control).

<table>
<thead>
<tr>
<th>Phylogenetic Markers</th>
<th>Sensitivity and phylogenetic markers for detecting Pseudomonas aeruginosa strains in animal product</th>
<th>Total number of samples tested</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>True Positives</td>
<td>False Negatives</td>
</tr>
<tr>
<td>RNA16S</td>
<td>32&lt;sub&gt;a&lt;/sub&gt;</td>
<td>7&lt;sub&gt;a&lt;/sub&gt;</td>
</tr>
<tr>
<td>rpoB</td>
<td>38&lt;sub&gt;a&lt;/sub&gt;</td>
<td>1&lt;sub&gt;a&lt;/sub&gt;</td>
</tr>
<tr>
<td>recA</td>
<td>34&lt;sub&gt;a&lt;/sub&gt;</td>
<td>5&lt;sub&gt;a&lt;/sub&gt;</td>
</tr>
<tr>
<td>ITS1</td>
<td>0.0&lt;sub&gt;b&lt;/sub&gt;</td>
<td>39&lt;sub&gt;b&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

Table 4: Sensitivity of the detection reaction by using phylogenetic markers for testing Pseudomonas aeruginosa strains in animal product. Each index letter indicates a subset of gene categories whose column proportions do not differ significantly from each other in level 05.

Sensitivity of different phylogenetic markers for stem tests

The sensitivity of the reaction with using four markers phylogenetic 16S RNAr, recA, rpoB and ITS1 for Pseudomonas aeruginosa identification indicates that a sensitivity of the rpoB housekeeping gene is 97.4% followed by the recA and 16S RNAr respectively 87.2% and 82.1% (Table 4) (Figures 13 to 15). However, no sensitivity reaction was observed with using ITS1 marker (Table 4 and Figure 16).
for PCR analysis by Urakawa [29]. The reference strain detection level *Pseudomonas aeruginosa* ATCC 27853 from the genes of households showed that with the *rpoB* gene, good resolution is obtained for a detection limit of 0.8 µg/ml. The best resolution of the *rpoB* gene could be due to the performance of the reaction with the species *Pseudomonas aeruginosa* [9,30]. The detection reaction by using 16S RNAr and *recA* has presented the same threshold of detection which is 7.6 µg/ml. This result could be explained by the fact that the RNAr 16S enables the identification of the genus *Pseudomonas*; while the *recA* gene allows identifying the species *Pseudomonas aeruginosa* and is most often associated with the 16S RNAr for complete identification [9,20,30].

For the positive controls CP2: 1125A and CP3: API, using of *rpoB* gene was also allow to get the best threshold of detection respectively 1.23 µg/ml and 0.1 µg/ml followed by *recA* 12.3 µg/ml and 0.9 µg/ml. For the positive controls CP2: 1125A and CP3: API, the using of *rpoB* gene was also allow to get the best threshold of detection respectively 1.2 µg/ml and 0.1 µg/ml followed by *recA* 12.3 µg/ml and 0.9 µg/ml. The results obtained with the RNAr 16S gene could be explained by the fact that 16S gene RNAr is the key molecule which is based on the classification of prokaryotes, including that of *Pseudomonas* [31].

Unlike to the study performed by Franzetti and Scarpellini [22], no threshold detection was observed with the ITS1 gene. This result discrepancy could be explained by the sensitivity and performance of the method used. Indeed, in order to increase the number of comparable characteristics, other authors have found that the ITS-PCR typing can be improved by digestion of the PCR products with the restriction endonucleases [32].

The study also tested the sensitivity of the three markers phylogenetic 16S RNAr, *recA* and *rpoB* for the identification of *Pseudomonas aeruginosa*. The sensitivity of the reaction with the housekeeping gene *rpoB* was 97.4% followed by the *recA* and 16S RNAr with 87.2% and 82.1% respectively. The high sensitivity by using *rpoB* gene could be justified by the fact that the functional genes such as *rpoB* could be used to target a more precise phylogeny and for a higher taxonomic resolution, because of its essential role in cellular metabolism [33]. This high positivity rate with *rpoB* gene also indicates that *rpoB* gene can be the best identifier tag of *Pseudomonas aeruginosa* species. These same results were obtained by Tayeb et al. [33] which stated in their study that the gene *rpoB* resolution gave a three (3) times greater than that obtained with the 16S rRNA gene.

However, ITS1 housekeeping gene showed no sensitivity to the presumptive identification of *Pseudomonas aeruginosa* strains. The study also showed that all test animal strains amplified with 16S rRNA genes (=1351 pb), *recA* (1041 pb) and *rpoB* (=759) were the same size as *Pseudomonas aeruginosa* ATCC 27853 reference strain and this confirms the membership of these animal strains to *Pseudomonas aeruginosa* species. Data from this study indicate that phylogenetic markers: 16S rRNA, *recA* and mainly *rpoB* used can be exploited for confirmation of *Pseudomonas aeruginosa* strains of different origin.

**Conclusion**

Data from this study indicate that phylogenetic markers: 16S rRNA, *recA* and *rpoB* used can be exploited for *Pseudomonas aeruginosa* strains confirmation of different origin. The study also showed that the sensitivity of the reactions was higher by using *rpoB* gene. This phylogenetic marker can therefore be recommended for the molecular identification of the *Pseudomonas aeruginosa* species.
References


