Comparison of Restriction Enzymes for Pulsed-Field Gel Electrophoresis Typing of *Moraxella catarrhalis*

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NotI, the most prevalent restriction enzyme used for typing *Moraxella catarrhalis*, failed to digest genomic DNA from respiratory samples. An improved pulsed-field gel electrophoresis (PFGE) methodology determined SpeI as the best choice for typing this bacterial species, with a good restriction of clinical samples and a good clustering correlation with NotI.

*M. catarrhalis* is a Gram-negative diplococcus commonly found colonizing the upper respiratory tract of children (1, 2). This mucosal pathogen is also a significant cause of recurrent otitis media in young children (3) and of acute exacerbations in chronic obstructive pulmonary disease (COPD) patients (3, 4), a syndrome associated with abnormal inflammatory immune responses of the lungs (5–7).

While working on a project on the dynamics of the microbial population in COPD, we attempted the genotypic characterization of *M. catarrhalis* isolates from sputum samples of patients attending the Pneumology Department. As the clinical interest for this microorganism is recent, typing systems are still being updated. In this study, 83 *M. catarrhalis* strains were isolated from 40 patients and identified by biochemical and microbiological techniques. Morphologically, *M. catarrhalis* has a butyrous consistency with colonies that can easily be slid across the agar surface (2, 8). This waxy surface is responsible for bacterial separation from the aqueous solution and could also be liable for the difficulties in bacterial lysis; this problem was solved by increasing the lysis steps to 20 h each, with an important improvement in the quality of the final digestion process (Fig. 1A and B). Other groups have also observed difficulties in cell lysis: Yano et al. (9) also incremented the lysis time, while recently Pingault et al. (10) increased EDTA and proteinase K concentrations.

After complete lysis of the bacterial cell, we encountered a second problem in the DNA digestion with the restriction endonuclease. Several pulsed-field gel electrophoresis (PFGE) typing methodologies have been reported (9–13), with NotI as the most prevalent enzyme and the best choice for genotyping *M. catarrhalis* isolates (9, 10, 12, 13). Nonetheless, our epidemiological study on clinical isolates from COPD patients showed that this enzyme was not a good option. Twenty-eight percent of the isolates (23/83) were not digested with the NotI restriction enzyme, probably due to the modification or methylation of the nucleosides present in the enzyme recognition sites. This problem was already reported in 1999 by Martinez et al. (11) in a molecular typing study performed on *M. catarrhalis* isolates of sputum samples from patients with respiratory infection; however, they used instead Smal, a restriction enzyme producing a small number of fragments that would not offer sufficient differentiation between isolates (11). The lack of a neoschizomere used to be a substitute for NotI encouraged the search for new restriction enzymes for typing *M. catarrhalis* clinical isolates.

This search was performed with the PFGE simulation provided at [http://insilico.ehu.es/](http://insilico.ehu.es/) (14), using the genome of *M. catarrhalis* RH4 (15) as the template. The selected enzymes produced a restriction pattern ranging from 16 to 31 bands (Table 1). To reduce the number of restriction enzymes to be tested and to ensure a proper digestion of the genomic DNA, we analyzed the possibility of activity blocking by methylation of substrate DNA by using the REBASE methylation sensitivity database (16) and comparing the results with those obtained for the NotI restriction enzyme. This analysis left us with three restriction enzymes: SpeI, SbfI, and PstI (the latter restriction enzyme could not be purchased). Cost-effectiveness of the restriction enzymes was evaluated by establishing the minimal amount (in units) of enzyme required for a complete digestion of the bacterial genome with respect to the discriminatory power of the enzyme. SbfI was eliminated, because a high number of units (>80 units) was necessary for complete DNA restriction (Fig. 1D1 and D2), leaving SpeI as the best choice for typing *M. catarrhalis* (Fig. 1C). Nevertheless, the discriminatory power of this enzyme had to be evaluated and compared to that of NotI, by using the updated PFGE methodology.

Molecular typing was performed on a bacterial suspension in 200 μl PIV (10 mM Tris·HCl, 1 M NaCl), diluted 1:200 and adjusted to a final optical density at 620 nm (OD 620) of 0.025. This bacterial suspension was thoroughly mixed with an equal volume of melted 1.5% low-melting-point agarose. DNA-agarose discs were incubated for 20 h at 37°C in ST buffer (6 mM Tris·HCl, 1 M NaCl, 0.1 M EDTA, 0.5% Brij-58, 100 μg/ml lysozyme, 50 μg/ml RNase), transferred into ES buffer (1 M EDTA, 1% sarcosyl, 1 mg/ml proteinase K), and incubated for 20 h at 50°C. The discs were rinsed three times with TE buffer (10 mM Tris·HCl, 1 mM EDTA).

Digestion of the DNA-embedded discs was performed following the manufacturer’s guidelines (New England BioLabs-IZASA, Barcelona, Spain) and by using (i) NotI (1 to 10 U), (ii) SpeI (1 to 10 U), and (iii) SbfI (20 to 100 U). DNA restriction fragments were separated in a 1% agarose gel with 0.5% TBE buffer (45 mM Tris base, 45 mM boric acid, 1.0 mM EDTA) in a contour-clamped homogenous electric field system (CHEF-DR-III; Bio-Rad) for 18 h at 14°C (6 V/cm and 120°)
and increasing pulse time intervals from 1 to 30 s (NotI) or 0.5 to 35 s (SpeI and SbfI). PFGE band patterns were analyzed using the Fingerprinting-II software, version 3.0 (Bio-Rad, Madrid), with optimization and tolerance for the Dice coefficient of 0.75%.

No restriction problems with SpeI were observed, even on the isolates that could not be digested with NotI. Fingerprinting analysis determined 20 different genotypes in the *M. catarrhalis* strain collection (Fig. 2). All the repeated samples showed the same

FIG 1 Differences in the quality of PFGE restriction analysis between 5 h of cell lysis with lysozyme and RNase (1 to 10 U NotI) (A) and 20 h of cell lysis (1 to 10 U NotI) (B). Cost-effectiveness of restriction enzymes after 20 h of cell lysis with 1 to 10 U of SpeI (C), 80 to 100 U of SbfI (D1), and 20 to 60 U of SbfI (D2). Numbers 1 to 4 represent four different *M. catarrhalis* isolates selected to test the minimum enzyme concentration required. Numbers I to IV represent enzyme concentration (NotI/ SpeI): I, 1 U; II, 2.5 U; III, 5 U; IV, 10 U. Numbers 20, 40, 60, 80, and 100 in panels D1 and D2 represent units of SbfI enzyme used per reaction.

<table>
<thead>
<tr>
<th>Restriction enzyme(s)</th>
<th>Restriction site</th>
<th>No. of cutting sites</th>
<th>$$/unit of enzyme</th>
<th>$$/digestion</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcII (Psp1406I)</td>
<td>5'-AA^CGTT-3'</td>
<td>19</td>
<td>0.21</td>
<td>ND</td>
<td>New England BioLabs</td>
</tr>
<tr>
<td>AcvI (BbrPI, Eco72I, PmaCI, PnII, PspCI)</td>
<td>5'-CAC^GTG-3'</td>
<td>27</td>
<td>0.031</td>
<td>ND</td>
<td>New England BioLabs</td>
</tr>
<tr>
<td>AgeI (AsiGI, BshTI, CspAI, PinAI)</td>
<td>5'-A^CCGGT-3'</td>
<td>23</td>
<td>0.22</td>
<td>ND</td>
<td>New England BioLabs</td>
</tr>
<tr>
<td>ApaI (Bsp120I)</td>
<td>5'-GGGCC^C-3'</td>
<td>22</td>
<td>0.013</td>
<td>ND</td>
<td>New England BioLabs</td>
</tr>
<tr>
<td>AsiSI (Rgal, SgfI)</td>
<td>5'-GGGAT^CGC-3'</td>
<td>18</td>
<td>0.126</td>
<td>ND</td>
<td>New England BioLabs</td>
</tr>
<tr>
<td>Bsp68I (BtuMI, NruI)</td>
<td>5'-TCG^CGA-3'</td>
<td>16</td>
<td>0.055</td>
<td>ND</td>
<td>New England BioLabs</td>
</tr>
<tr>
<td>BsrNI (Eco105I, SnaBI)</td>
<td>5'-TAC^GTA-3'</td>
<td>16</td>
<td>0.11</td>
<td>ND</td>
<td>New England BioLabs</td>
</tr>
<tr>
<td>NotI</td>
<td>5'-GC^GGCGGC-3'</td>
<td>12</td>
<td>0.136</td>
<td>1.36</td>
<td>New England BioLabs</td>
</tr>
<tr>
<td>PfoI</td>
<td>5'-T^CCNGGA-3'</td>
<td>19</td>
<td>0.12</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SfoI (BbeI, Kad, NarI)</td>
<td>5'-GGC^GCC-3'</td>
<td>27</td>
<td>0.12</td>
<td>ND</td>
<td>New England BioLabs</td>
</tr>
<tr>
<td>SpeI (AhlI, BcuI)</td>
<td>5'-A^CTAGT-3'</td>
<td>31</td>
<td>0.126</td>
<td>0.126</td>
<td>New England BioLabs</td>
</tr>
<tr>
<td>SbfI (SdaI, Sse8387I)</td>
<td>5'-CCTGCA^GG-3'</td>
<td>28</td>
<td>0.134</td>
<td>10.72</td>
<td>New England BioLabs</td>
</tr>
<tr>
<td>Pasi</td>
<td>5'-CC^CWGGG-3'</td>
<td>24</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Restriction enzymes in parentheses recognize the same sequence. Underlined restriction enzymes are neoschizomer (recognize same sequence but cleave at different positions).

b The cost was calculated on the basis of the prices indicated by the manufacturer (listed in 2012).

c NotI, restriction enzyme previously used in PFGE analysis of *M. catarrhalis*.

d ND, not determined.

e ^, cleavage site.
FIG 2 Dendrogram (generated using Fingerprinting software) based on PFGE variation of 83 *M. catarrhalis* strains with the SpeI restriction endonuclease. The dotted black line indicates the limit for a coefficient of similarity of ≥80% between strains that were considered to belong to the same cluster.
PFGE pattern, which corroborates the reproducibility of this updated methodology.

The evaluation of the discriminatory power of SpeI with respect to that of NotI was performed on 35 nonrepeated isolates from different episodes of 31 patients with COPD that presented a complete digestion for NotI. As shown in Fig. 3, the discriminatory power of NotI (19 genotypes) is slightly superior to that of SpeI (14 genotypes). A previous study published by Vu-Thien et al. (13) on 11 *M. catarrhalis* isolates suggested that although NotI had the most discriminatory power, SpeI patterns were also reliable. Our study, performed on 35 independent isolates, proves that the difference between both restriction enzymes is minimal (Fig. 3) and, probably, the importance of a correct restriction of all the samples outweighs the small difference in discriminatory power.

A methodology for PFGE should be cost-effective and standardized to allow the comparison of fingerprints between laboratories. So far, genotyping of *M. catarrhalis* involved different restriction enzymes, and the epidemiological results could not be compared between centers. The NotI restriction enzyme proved to be inefficient, at least for typing *M. catarrhalis* isolated from respiratory samples. The evaluation indicated that PFGE with SpeI is almost as discriminatory as the one performed with NotI, and so far it avoids the restriction problems encountered in respiratory samples.

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We report no transparency declarations.

REFERENCES
