Real-Time PCR for Determining Capsular Serotypes of *Haemophilus influenzae*<sup>▼</sup>

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A two-step real-time PCR assay targeting all six capsulation loci of *Haemophilus influenzae* (i.e., serotypes a to f) was developed and compared with a previously published qualitative PCR assay by using 131 *H. influenzae* clinical isolates. There was a 98.5% concordance between the two tests. The sensitivity of detection of capsular type-specific reference strains of *H. influenzae* a to c (10⁴ CFU/PCR) was higher than that for type e (10³ CFU/PCR) and types d and f (10⁴ CFU/PCR), and a broader dynamic range was obtained (5 to 8 log₁₀ units). No cross-reaction was observed with bacteria commonly isolated from the respiratory tract. We showed that both PCR assays are more reliable than slide agglutination serotyping. The real-time PCR-based assay seems to be an alternative of choice for the epidemiological follow-up of *H. influenzae* invasive infections.

*Haemophilus influenzae* is a major pathogen causing a variety of infections, from acute and chronic respiratory diseases to meningitis and sepsis. The *H. influenzae* capsule is a major virulence factor (8, 11). Currently, *H. influenzae* is identified biochemically, and the capsular type is determined by the slide agglutination test (2), but the latter is not fully reliable (13). The amplification of genes involved in capsule expression has opened new ways to rapid, specific, and highly sensitive detection (5, 6, 22). Recently, a real-time quantitative PCR-based assay was evaluated for the detection of *H. influenzae* strains, using the *cap* locus (15, 16), the capsule-producing gene (*bexA*) (3, 18), the 16S rRNA gene (16, 17, 23), the insertion-like sequence (*IS1016*) (16), and the outer membrane protein D gene (*glpQ*) (20) as targets. We developed a new highly specific and sensitive real-time PCR procedure for typing all existing *H. influenzae* capsules.

Positive controls were *H. influenzae* strains ATCC 9006 (type a), ATCC 9795 (type b), ATCC 9007 (type c), ATCC 9332 (type d), ATCC 8142 (type e), and ATCC 9833 (type f). The specificity of the real-time PCR assay for *H. influenzae* was tested with *Streptococcus pneumoniae* (ATCC 33400), *Streptococcus oralis* (ATCC 10557), *Staphylococcus aureus* (ATCC 65389), *Staphylococcus epidermidis* (ATCC 12722), *Staphylococcus haemolyticus* (ATCC 29970), *Escherichia coli* (ATCC 35039), *Enterococcus faecalis* (ATCC 29212), *Haemophilus parainfluenzae*, *Haemophilus haemolyticus*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Moraxella catarrhalis*, and *Moraxella lacunata*. Clinical strains isolated from blood, bronchoalveolar lavage (BAL) fluid, and sputa from patients aged less than 1 day to 84 years old were sent to the reference laboratory (Institut Jules Bordet) by Belgian microbiologists during 2004 and 2005 (n = 131). Capsular serotyping was performed by slide agglutination (with antisera against capsular antigen [Difco *Haemophilus influenzae* antisera]). A strong and rapid reaction with only one antiserum was required to record a positive test without autoagglutination.

DNAs were extracted as previously described (14), using a QIAamp DNA blood kit (QIAGEN, Hilden, Germany). Primers and probes designed for the detection of *H. influenzae* capsular types a to f (*Hia* to *Hif*) were targeted within variable region II of the encapsulation *cap* locus (21). For the *bexA* gene, several sequence variations have been reported (10, 12, 18, 19). We used the MegAlign program of DNASTAR, version 5.07 (Madison, WI), to compare 18 published *bexA* sequences (National Center for Biotechnology Information [NCBI] GenBank sequence database), and we chose a primer-probe combination situated in the most conserved regions. Moreover, one primer and probe set derived from the sequence for the gene coding for outer membrane lipoprotein P2 (*ompP2*), present in both encapsulated and noncapsulated (NC) *Haemophilus* strains, was also designed as a control for real-time PCR to confirm the *H. influenzae* species. The oligonucleotide sequences, PCR product lengths, locations, and GenBank accession numbers for the corresponding target genes are displayed in Table 1. Prior to experimental testing, these sequences were assessed for specificity by comparing them to sequences of other prokaryotic and eukaryotic organisms, using standard nucleotide-nucleotide BLAST (NCBI) alignment software. None of the selected oligonucleotides had significant homology to any other sequences. All primers and probes included in this study were designed using Primer Express software, version 2.0 (Applied Biosystems, Foster City, CA), and were synthesized by Invitrogen (Merelbeke, Belgium) and Applied Biosystems, respectively.

The following five conventional and three minor groove binder (MGB) fluorogenic probes, with a reporter dye on the 5’ end and a quencher (nonfluorescent quencher [NFQ] or black hole quencher [BHQ]) on the 3’ end, were used in this study: *bexA*-Cy3 (where Cy3 is indocarbocyanin), *Hia*-FAM (where FAM is 6-carboxyfluorescein), *Hib*-JOE (where JOE is 6-carboxy-4’,5’-dichloro-2’,7’-dimethoxyfluorescein), *Hic*-VIC, *Hid*-NED (Applied Biosystems), *Hie*-FAM, *Hif*-Cy3, and *ompP2*-FAM, used for the detection of *bexA*, *Hia*, *Hib*, *Hic*, *Hid*, *Hif*, and *ompP2* DNAs, respectively (Table 1). The
optimal concentration of oligonucleotides used in real-time PCR was assessed by using primer and probe optimization matrices along with serial PCRs across concentration ranges from 50 to 900 nM and 50 to 250 nM, respectively. The oligonucleotide concentration which gave the lowest cycle threshold \( C_T \) value and the maximum amplification efficiency was selected (Table 1).

Strains were first tested for the presence or absence of the \( bexA \) gene, region II of the \( cap \) locus of Hib, and the \( ompP2 \) gene by a multiplex real-time PCR-based assay. The \( ompP2 \) gene was used as an internal control for DNA extraction and amplification. Non-b capsulated \( H. influenzae \) strain 151 was used as an internal control for DNA extraction and amplification. The sensitivities and detection efficiencies of the real-time PCR assays were performed using an ABI Prism 7500 sequence detection system (Applied Biosystems). For multiplex amplification, the cycling parameters were 95°C for 2 min and 95°C for 10 min, followed by 40 cycles comprising a denaturation step of 95°C for 15 s and annealing and primer extension at 58°C for 1 min. For duplex PCR, covering Hib and Hif, the same conditions were used, except for an annealing temperature of 60°C. Lastly, for uniplex amplifications, annealing temperatures were 58°C (types c and d) and 56°C (type e). Finally, conventional gel-based PCR capsular typing was performed by the method described by Falla et al. (5).

The sensitivities and detection efficiencies of the real-time PCR assays were assessed by repeated testing of 10-fold serial logarithmic dilutions of reference bacterial strains, starting from cultures of \( 10^8 \) CFU/ml. This was achieved by plotting the number of cycles necessary in each of the real-time PCRs to produce a fluorescence signal exceeding a threshold limit against the \( \log_{10} \) of the number of microorganisms. All dilution series of Hib to Hif yielded similar regression lines, as follows: the amplification efficiencies were \(-3.200, -3.107, -3.106, -3.117, -3.305, \) and \(-3.151\), respectively; the reproducibility and consistency of the replicates in real-time PCRs were 0.983, 0.988, 0.980, 0.994, 0.987, and 0.989, respectively; and the theoretical limits of detection of the reactions were 42.306, 39.232, 43.742, 48.672, 47.485, and 47.769, respectively. The actual sensitivities of the PCRs, referred to as the lowest standard dilutions constantly producing real-time amplification signals

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**TABLE 1. Primers and probes for capsule type-specific \( H. influenzae \) detection by real-time PCR**

<table>
<thead>
<tr>
<th>Capsular type</th>
<th>Amplicon length (bp)</th>
<th>Oligonucleotide</th>
<th>Oligonucleotide sequence (5'→3')</th>
<th>Probe label</th>
<th>T&lt;sub&gt;m&lt;/sub&gt; (°C)</th>
<th>Concen (nM)</th>
<th>Nucleotide positions</th>
<th>GenBank accession no.</th>
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<tbody>
<tr>
<td>Hia 83</td>
<td>Forward primer</td>
<td>GCAACCATCTTCATACATTCAGGA</td>
<td>ATAC ATAC ATAC ATAC ATAC ATAC</td>
<td>FAM BHQ1</td>
<td>60 900</td>
<td>2300–2326</td>
<td>Z37516</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>AATAGTGGTGAATATCCATTCAGGA</td>
<td>GGTCGCGGTTGCCTGTTGTT</td>
<td>JOE BHQ1</td>
<td>70 250</td>
<td>2331–2356</td>
<td>X78559</td>
<td></td>
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<tr>
<td>Hib 147</td>
<td>Forward primer</td>
<td>TTGTTGCGATAATCTTTCATTTAG</td>
<td>CACAAAATCTTCTATTTTCTTCTGAG</td>
<td>JOE BHQ1</td>
<td>59 900</td>
<td>5579–5602</td>
<td>X78559</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>CTTACGCTTCTATTCGTTGATTAA</td>
<td>CCTA CCTA CCTA CCTA CCTA</td>
<td>JOE BHQ1</td>
<td>59 900</td>
<td>5725–5698</td>
<td>X78559</td>
<td></td>
</tr>
<tr>
<td>Hic 67</td>
<td>Forward primer</td>
<td>TCGTTGATTAGTATGTTGCTAGTAG</td>
<td>TGACGTTAAGATTATTATTAGTTA</td>
<td>VIC MGBNFQ</td>
<td>55 900</td>
<td>34–57</td>
<td>Z33387</td>
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<tr>
<td></td>
<td>Reverse primer</td>
<td>TTAGGATATTTACGCTGCAATTAG</td>
<td>CTGACTTTTTTCTTTCCTTCTT</td>
<td>VIC MGBNFQ</td>
<td>67 125</td>
<td>59–74</td>
<td>Z33387</td>
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<tr>
<td>Hid 120</td>
<td>Forward primer</td>
<td>TATTGATGAGCTGATACCTCTTGA</td>
<td>TAAATGTTGGAATACCTTCTTTCTT</td>
<td>NED MGBNFQ</td>
<td>60 900</td>
<td>100–79</td>
<td>Z33387</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>AATTGTTGGAATACCTTCTTTCTT</td>
<td>TTTAGTTGGAATACCTTCTTTCTT</td>
<td>NED MGBNFQ</td>
<td>60 900</td>
<td>4–31</td>
<td>Z33387</td>
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<tr>
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<td>GTGAAATACGACATCTTTTCTTTT</td>
<td>AATAGTTGGAATACCTTCTTTCTT</td>
<td>MGBNFQ</td>
<td>58 900</td>
<td>1–21</td>
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<tr>
<td></td>
<td>Reverse primer</td>
<td>ATCTTTAATACGCTACCTCTTCTTCTT</td>
<td>ATCTTTAATACGCTACCTCTTCTT</td>
<td>MGBNFQ</td>
<td>59 900</td>
<td>73–48</td>
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<tr>
<td>Hib 125</td>
<td>Forward primer</td>
<td>GGATATCATAATTCCACAATTCTGCTTTT</td>
<td>TCATCGTTGAGATCTGATCTGAT</td>
<td>CY3 BHQ2</td>
<td>58 900</td>
<td>8121–8146</td>
<td>AF549211</td>
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<tr>
<td></td>
<td>Reverse primer</td>
<td>TGTAGTATGCTGTTAATACATGTTGAA</td>
<td>TTGTTGGAATACCTTCTTTCTT</td>
<td>CY3 BHQ2</td>
<td>58 900</td>
<td>8178–8202</td>
<td>AF549211</td>
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<tr>
<td>bexA 101</td>
<td>Forward primer</td>
<td>GTGAATRGGGATTTCTTCTTATTA</td>
<td>TTTTAGTTGGAATACCTTCTTTCTT</td>
<td>MGBNFQ</td>
<td>59 900</td>
<td>358–382</td>
<td>M19995</td>
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<tr>
<td></td>
<td>Reverse primer</td>
<td>AGGTGTTAGCTGGCCTGTTGCTAG</td>
<td>TTTTAGTTGGAATACCTTCTTTCTT</td>
<td>MGBNFQ</td>
<td>59 900</td>
<td>404–426</td>
<td>M19995</td>
<td></td>
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<tr>
<td>ompP2 151</td>
<td>Forward primer</td>
<td>GTGTGCATCCAGCAGCTTTCA</td>
<td>TGTTTAGTTGGAATACCTTCTTTCTT</td>
<td>JOE BHQ1</td>
<td>58 300</td>
<td>458–435</td>
<td>CP000057</td>
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<tr>
<td></td>
<td>Reverse primer</td>
<td>ACAGTAAATCACAGCCAGCGCTTTCA</td>
<td>ATCTTTAATACGCTACCTCTTCTT</td>
<td>JOE BHQ1</td>
<td>68 500</td>
<td>215217–215235</td>
<td>CP000057</td>
<td></td>
</tr>
</tbody>
</table>

* R, A or G; Y, C or T; H, A, T, or C.
in replicate reactions, were $10^1$ CFU per PCR for types a, b, and c; $10^2$ CFU per PCR for type e; and $10^3$ CFU per PCR for types d and f. The intra- and interassay variabilities of the C<sub>T</sub> values obtained with replicates of the same DNA extracted from each dilution series or with replicates from DNAs extracted from different dilution series were <1 (data not shown). Each *H. influenzae* capsule type-specific primer/probe system correctly identified its corresponding target strain, without cross-reaction with purified DNAs from heterologous species (the bacteria mentioned above). The primer/probe system specific for the bexA gene amplified only encapsulated strains; NC *H. influenzae* strains and nontarget microorganisms were not detected.

One hundred thirty-one *H. influenzae* isolates were tested using the real-time PCR-based approach. All strains yielded at least the ompP2 signal. Of these isolates, 9.16% (12/131 samples) displayed the bexA signal and were subsequently identified by the real-time PCR assay as capsule type b, e, or f (Table 2), while 90.84% (119/131 isolates) of isolates did not display the bexA signal. These results were compared to those established by the slide agglutination test and gel-based PCR. Overall, there were 117 NC isolates by both PCR methods and nontypeable (NT) isolates by the slide agglutination test. Among the eight Hib strains typed by real-time PCR, three blood isolates (from unvaccinated patients 1, 2, and 4) and one BAL isolate (from patient 3) were fully concordant with both other methods, three blood isolates were concordant with the gel-based PCR result but NT by the slide agglutination test (patients 9 to 11), and one BAL isolate was NT by both slide agglutination and gel-based PCR (patient 8) (Table 2). One each of Hie (patient 5) and Hif (patient 6) isolates was fully concordant with all methods, and one was concordant with the slide agglutination test but NC by gel-based PCR (Hie blood isolate from patient 7) or concordant with the gel-based PCR result but NT with the slide agglutination test (Hif BAL isolate from patient 12). Two NC isolates by both PCR methods, both isolated from blood, were typed with the slide agglutination test as Hia (from patient 13) and Hie (from patient 14). The overall agreement rate between all three methods was therefore 93.89% (123/131 isolates), and that between real-time PCR and serotyping was 94.7% (124/131 isolates). As indicated above and shown in Table 2, serotyping results and real-time PCR results did not agree for seven isolates, four of which were recognized as type b, one of which was type f, and two of which were NC by PCR.

Our study showed that both the real-time and the gel-based PCR assays were more sensitive than the slide agglutination test for capsular typing of *H. influenzae*, as reported by others (1, 6, 13). Among our strains, 6% were Hib by real-time PCR, 5.4% were Hib by gel-based PCR, and 3% were Hib by serotyping. Only two cases of discordance were observed between the two PCR assays (98.5% concordance between both PCR methods): one BAL isolate typed as Hib by real-time PCR could not be typed by any other method (from patient 8, a 4-year-old boy with recurrent pulmonary infections), and one blood isolate typed as Hie by real-time PCR and the slide agglutination test could not be typed by the gel-based PCR assay (from patient 7, a 54-year-old woman [no clinical information available]). The fluorescence intensities of these isolates in the real-time PCR assay were weak (average $C_T$ value, 35.5), indicating possible misidentification by conventional PCR. An increased sensitivity of real-time PCR compared to that of gel-based PCR was expected because of the probable higher efficacy of amplification reactions under short real-time cycling conditions and the quality of both TaqMan PCR master mixes and the polymerase used in real-time PCR (4). Notably, in this study period, no capsule-deficient mutant Hib strains (b- strains) of *H. influenzae* were detected by either amplification assay. Although the number of isolates studied was too small to be conclusive, the findings suggest that the incidence of invasive b- strains of *H. influenzae* is low in Belgium.

The strategy of identification of *H. influenzae* presented here, using a capsular typing scheme targeting all *H. influenzae* serotypes, could be used as a novel nonculture method for typing of *H. influenzae*. Compared to gel-based PCR, the new assay has the following added benefits: it includes an internal control for extraction and amplification (ompP2), it can be adapted in a quantitative format if needed, and it has a shorter...
turnaround time (1.5 h) than that of conventional PCR (5 to 6 h). On the other hand, due to its relatively low diagnostic sensitivity, poor specificity, and subjective reading, the serological determination of capsular type by the slide agglutination test can no longer be recommended for the workup of samples requiring precise and unequivocal identification, such as *Haemophilus influenzae* strains infecting immunized children. In the future, the development of a method with only two multiplexed real-time PCRs covering all capsular types, with the ability to use uniform PCR conditions, would be useful for economizing reagents and accelerating the diagnostic analysis.

REFERENCES


