Evaluation of four commercial real-time PCR assays for the detection of *Bordetella* in nasopharyngeal aspirates

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Abstract

We evaluated the performance of 4 commercial real-time PCR kits for *B. pertussis* IS481 sequence detection in nasopharyngeal aspirates, by comparison with an in-house real-time PCR assay. Among them, Simplexa Bordetella pertussis-parapertussis (Focus diagnostics), SmartCycler Bordetella pertussis/parapertussis Assay (Cepheid) and Bordetella-R-gene (Argene) present a sensitivity over 90%. One kit proved unsuitable for routine clinical use.
Nucleic acid amplification tests (NAAT), including PCR and, more recently, real-time PCR, overcome some of the limitations of culture and serological methods for the diagnosis of *Bordetella* infection (1, 6, 12). NAAT targets include IS481 for *B. pertussis* (gene also present in *B. holmesii* and sometimes in *B. bronchiseptica*) (12, 13), the pertussis toxin promoter or porin gene or Bp 3385 for *B. pertussis* (10), and IS1001 for *B. parapertussis* (5, 13). NAAT methods are highly sensitive and may be either genus- or species-specific, depending on the choice of primers and targets. Real-time PCR accelerates the diagnostic process by combining amplification and detection (12, 13). These methods have proved more sensitive than the equivalent gel-based system (9, 12) and many medical laboratories have developed in-house tests. A voluntary external molecular quality control procedure for these in-house methods was set up in France in 2009 by the National Reference Centre for Whooping Cough and other Bordetelloses (Institut Pasteur, Paris) (3), following a study performed in eight hospital laboratories throughout France to assess the performance of in-house methods and adaptations of the techniques developed by Reischl *et al.* (11), Kosters *et al.* (8) and Templeton *et al.* (3, 13). Commercial molecular diagnostic kits are now available, but have never been comparatively tested.

The aim of this study was to compare the performance of 4 commercial real-time PCR assays for the detection of *B. pertussis*, using as reference an in-house method evaluated during the French external molecular quality control (3).

Eighty-one non-redundant nasopharyngeal aspirates (NPA) from patients with suspected pertussis were tested in the bacteriology laboratories of three French teaching hospitals (Tours, Poitiers and Limoges). Sixty-six samples tested positive with our in-house PCR, while 15 had tested negative but were from patients with a suspicion of pertussis. The samples and DNA extracts were stored at -20°C until use. DNA was extracted with the Invisorb Spin Cell Mini kit®.
(Invitek, Germany) in Tours, and with the QIAamp DNA Mini kit® (Qiagen) in Poitiers and Limoges, following the manufacturers’ instructions. Principles and procedures are very similar between the two extraction methods. Ten-fold serial dilutions of DNA from *B. pertussis* strain Tohama, containing 238 copies of IS481, were used to determine the analytical sensitivity of each method. DNA extracted from each of the 81 samples was tested with the in-house real-time PCR assay using primers Bp481F (5’-CCGAACCGGATTTGAGAAAC-3’) and BP481R (5’-TAGGAAGTCAATCGGGCAT-3’) that target a 100-bp fragment of IS481. Detection was based on hybridization of the Bp481S probe (5’FAM-CCGGCCGGATGAACACCTAATAA-3’ TAMRA). Primers were purchased from Eurogentec® (Seraing, Belgium) and were used at a concentration of 10 µmol/L. Amplification was performed with a Smart Cycler® device (Cepheid, Maurens-Scopont, France), with 5 µL of DNA, 12.5 µL of Premix Ex Taq® (TaKaRa, Foster city, USA), 4.2 µL of water, 0.4 µL of each primer and 2.5 µL of Bp481S probe (1 µM). The thermal cycling conditions were as follows: 1 cycle of 15 s at 95°C, followed by 45 cycles of 5 s at 95°C, and 10 s at 60°C. Each laboratory tested the specimens collected in its host hospital and used the same protocols. The Smart Cycler II apparatus (Cepheid) was used for all assays.

The four commercial PCR kits, all based on Taqman technology, were Bordetella R-gene® (Argene, Verniolle, France), Simplexa Bordetella pertussis/parapertussis® (Focus, distributed in France by Eurobio, Courtabeuf, France), Bordetella pertussis Real Time PCR kit® (Cat N° RD-0061-02, Shanghai ZJ Bio-Tech, distributed in France by Bioadvance, Bussy Saint Martin, France) and SmartCycler Bordetella pertussis/B. parapertussis Assay® (Cepheid). The different kits were used as recommended by the manufacturers, including cycle thresholds (Ct) of 7 for the Shanghai ZJ Bio-Tech kit, 15 and 30 for the Focus kit, and 30 for the other two methods. Differences between the methods are shown in Table I.

The analytical sensitivity of each method, tested in duplicate, is shown in figure 1. The Ct values were similar with the different methods up to 104 copies of IS481 per reaction, except that the Ct of the Shanghai ZJ Bio-tech test was about 7 cycles above that of the other methods whatever the
number of copies. Only the in-house method and the Argene, Cepheid and Focus kits were positive with the dilution containing 100 copies of IS481 per reaction.

Each clinical DNA specimen was tested with the in-house method and with the 4 commercial kits. All the DNAs were thawed and tested on the same day (no refreezing between the different assays). The results are shown in Table II. No false positives were noted for any assay. With the in-house method as the gold standard, clinical sensitivity was 97% with the Focus kit, 93.9% with the Cepheid kit, 90.9% with the Argene kit, and 51.5% with the Shanghai ZJ Bio-tech kit. The Focus kit had the lowest mean Ct (Table II), which was not significantly different from that of the in-house assay. The Argene and Cepheid kits had slightly but not significantly higher mean Ct values than the in-house assay. In contrast, the Shanghai ZJ Bio-Tech kit had significantly higher mean Ct values than the in-house assay. It is noteworthy that the manufacturer of the Shanghai ZJ Bio-tech kit recommends using only half the amount of DNA used in the other kits. The performance of the Focus kit was unaffected when a threshold of 15 Ct rather than 30 Ct was used (data not shown).

Internal controls are included to detect PCR inhibition. Beta-globin was used for this purpose in the in-house method in a separate vial. The Argene and Focus kits also include extraction controls (Table I). The list of apparatus on which kits can be used is shown in Table I. Two kits (Cepheid and Focus) are claimed to detect *B. pertussis* and *B. parapertussis* in the same tube, while the other two kits and the in-house method only target IS481. *B. holmesii*, which also possesses IS481, cannot be distinguished from *B. pertussis* with these kits. This species rarely induces pertussis-like symptoms and can be identified by real-time PCR-based on *recA* gene (7).

In conclusion, all four kits tested here were highly specific, whereas their sensitivity was highly variable. The Argene, Focus and Cepheid kits performed as well as the in-house method in terms of both analytical and clinical sensitivity. These three kits include an internal control to detect inhibitors, which is important in routine practice (4), but only two of them, Argene and Focus,
validate the extraction step. The other kit needs to be improved if they are to be used in routine clinical settings.

Acknowledgments

The authors thank Marie Odile Viaud, Lucie Yzon and Catherine Louin for their technical assistance. The different kits were supplied free of charge by the different corporations, which provided no other financial support for this study.
References


Figure 1. Representation of the crossing threshold obtained for each commercial kit and the in-house real-time PCR for the detection of *Bordetella* IS481 sequence.
Table I. Comparison of the main parameters of the 4 Real-Time PCR kit tested

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Name of the kit</th>
<th>Target pathogens</th>
<th>Target genes</th>
<th>Mix containing primers and probe ready-to-use</th>
<th>Internal control (IC) detection</th>
<th>Extraction controls</th>
<th>positive control available in the kit</th>
<th>Reagents storage</th>
<th>Final reaction vol (µl)</th>
<th>DNA from sample added vol (µl)</th>
<th>No. of cycles used for the comparison (No. of cycles (manufacturer))</th>
<th>No. of tests by kit</th>
<th>Kit available on the following apparatus</th>
</tr>
</thead>
<tbody>
<tr>
<td>In-house</td>
<td>-</td>
<td>B. pertussis</td>
<td>IS481</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>-</td>
<td>-20°C</td>
<td>20</td>
<td>5</td>
<td>45 (45)</td>
<td>-</td>
<td>LightCycler (LC480/LC2.0), SmartCycler, ABI Prism,Rotor-gene</td>
</tr>
<tr>
<td>Argene</td>
<td>Bordetella-R-Gene</td>
<td>B. pertussis</td>
<td>IS481</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>-20°C</td>
<td>25</td>
<td>10</td>
<td>45 (45)</td>
<td>60</td>
<td>LightCycler (LC480), i-cycler (IQ4/IQ5), SmartCycler II, ABI</td>
</tr>
<tr>
<td>Changzhou ZJ Bio-Tech</td>
<td>(distributed in France by Bioadvance)</td>
<td>B. pertussis - real-time PCR kit</td>
<td>IS481</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>-20°C</td>
<td>25</td>
<td>2.5</td>
<td>45 (40)</td>
<td>25</td>
<td>LightCycler (LC480), i-cycler (IQ4/IQ5), SmartCycler II, ABI</td>
</tr>
<tr>
<td>Copheid</td>
<td>SmartCycler Bordetella pertussis/parapertussis Assay</td>
<td>B. pertussis &amp; B. parapertussis</td>
<td>IS481 (Fp), IS100 (Bpp)</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>4 - 4°C (beads)</td>
<td>25</td>
<td>5</td>
<td>45 (40)</td>
<td>40</td>
<td>SmartCycler II</td>
</tr>
<tr>
<td>Focus Diagnostics</td>
<td>Simplexa Bordetella pertussis-parapertussis</td>
<td>B. pertussis &amp; B. parapertussis</td>
<td>IS481 (Fp), IS100 (Bpp)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>-20°C</td>
<td>25</td>
<td>5</td>
<td>45 (45)</td>
<td>48 LightCycler (LC480), SmartCycler II, ABI 7500</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

*as recommended by the manufacturer for the SmartCycler apparatus*
Table II. Clinical sensitivities and mean Ct values of the five Real-Time PCR kits tested in comparison to results of in-house real-time PCR for detection of *Bordetella* IS481 sequence in 66 positive *Bordetella* specimens

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Number of positive specimens</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Mean Ct +/- SD</th>
<th>P value ***</th>
</tr>
</thead>
<tbody>
<tr>
<td>In-house</td>
<td>66/66</td>
<td>100</td>
<td>100</td>
<td>25.5 +/- 8.6</td>
<td>ns</td>
</tr>
<tr>
<td>Argene kit</td>
<td>60/66</td>
<td>90.9</td>
<td>100</td>
<td>27.1 +/- 9.5</td>
<td>ns</td>
</tr>
<tr>
<td>Cepheid Kit</td>
<td>62/66</td>
<td>93.9</td>
<td>100</td>
<td>25.8 +/- 9.2</td>
<td>ns</td>
</tr>
<tr>
<td>Focus Diagnostics kit</td>
<td>64/66</td>
<td>97</td>
<td>100</td>
<td>24.8 +/- 8.8*</td>
<td>ns</td>
</tr>
<tr>
<td>Shanghai ZJ Bio-Tech kit</td>
<td>34/66</td>
<td>51.5</td>
<td>100</td>
<td>37.3 +/- 8**</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

* As recommended by the manufacturer, the Ct retained for the comparison was 15

** As recommended by the manufacturer, the Ct retained for the comparison was 7

*** paired t-test (2)