Comparison of Serological and Real-Time PCR Assays To Diagnose Bordetella pertussis Infection in 2007

Philippe André,1 Valerie Caro,2 Elisabeth Njamkepo,2 Aaron M. Wendelboe,3 Annelies Van Rie,3 and Nicole Guiso*4

Sanofi Pasteur, 2 Avenue Pont Pasteur, 69367 Lyon Cedex 07, France; Institut Pasteur, Unité Prévention et Thérapie Moléculaires des Maladies Humaines, URA-CNRS 3012, 25 rue du Dr Roux, 75015 Paris, France; and Department of Epidemiology, School of Public Health, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina3

Received 13 November 2007/Returned for modification 17 December 2007/Accepted 18 March 2008

Bacterial culture for diagnosing pertussis infection has high specificity but poor sensitivity and is slow. Highly sensitive real-time PCR assays and single-serum pertussis serology have been developed to overcome these limitations, but there are few data available on the relative sensitivities and specificities of such assays for pertussis diagnosis. Using data on 195 participants (≥7 years old) from an epidemiological study, we assessed the sensitivity, specificity, and performance (Youden index) for pertussis diagnosis of the pertussis toxin (ptxA) -specific PCR assay (using single and paired serology) and of real-time PCR assays (using the IS481 and ptxA-Pr targets). All available diagnostic information (clinical and laboratory) was pooled to serve as the gold standard. Single serology was the most efficient diagnostic test (Youden index, 0.57 to 0.58), with relatively high sensitivity (>64%) and high specificity (>90%), independent of the cutoff level. IS481 PCR performance was superior to that of ptxA-Pr PCR, and it was the second-most-efficient tool (Youden index, 0.30). Performing both ptxA-Pr and IS481 PCR did not improve diagnostic performance. The greatest test efficiency (Youden index, 0.69 to 0.74) was achieved when single-serum serology was used in combination with IS481 or ptxA-Pr PCR or paired serology. Combining single serology with one PCR or paired serology increased the sensitivity with an associated limited decrease in specificity. The most specific tests for diagnosis of pertussis were single serology and ptxA-Pr PCR, and the most sensitive diagnostic tool was the combination of IS481 PCR with single serology.

Currently there is no satisfactory gold standard technique for laboratory confirmation of a pertussis infection. Although culture is highly specific, sensitivity is low and declines with the duration of illness, and the method may take up to 7 days to provide a result. Using culture alone to diagnose pertussis is likely to lead to underreporting of pertussis cases (8). Serological assays, using one or two serum samples, have been developed to improve the sensitivity of the pertussis diagnosis, but they have a lower specificity than culture (3, 9, 20, 25). PCR techniques were developed to overcome these limitations, but despite a consensus meeting in 2005 (24), these techniques still require further standardization and optimization. To date there are few reports (8) in which the sensitivities and specificities of these different techniques are compared using a population exposed to pertussis.

The objective of this study was to compare the sensitivities and specificities of the current most widely used techniques to diagnose pertussis in exposed populations, namely, two PCR methods (IS481 and ptxA-Pr targets) and single and paired serology for detection of antipertussis toxin (PT) antibodies in the serum of the patients using enzyme-linked immunosorbent assay (ELISA) with two different cutoffs (two- or fourfold change for paired serology and ≥100 or ≥125 ELISA units [EU]/ml for single serology).

MATERIALS AND METHODS

Patients and specimens. We used data obtained in the course of a recent prospective multicenter epidemiologic study (27). This study included 404 household contacts of 94 young infants (≥6 months of age) with laboratory-confirmed pertussis. All contacts were interviewed face-to-face using a standard questionnaire to obtain relevant demographic and clinical data, particularly the presence and duration of symptoms of cold or cough during the month prior to their inclusion. They also provided nasopharyngeal aspirates or, in a few centers, swab samples for culture and PCR detection of Bordetella pertussis and an acute blood sample for detection of anti-PT immunoglobulin G (IgG). One month later, data on the presence of cough and cold-like symptoms and a convalescent-phase blood sample were collected from participating contacts. All nasopharyngeal aspirates, swabs, and sera were sent to our laboratory for analysis with real-time PCR (using the IS481 target or the ptxA-Pr target) and measurement of anti-PT IgG by ELISA.

Culture and analysis of isolates. Culture was performed in only a few participating centers, resulting in five isolates from France, three from Germany, and five from the United States. Isolates were analyzed by pulsed-field gel electrophoresis (PFGE) and genotyping (7) and expression of bacterial toxins and adhesins analyzed using specific polyclonal and monoclonal antibodies (16, 22).

PCR. (i) Detection of B. pertussis by real-time PCR targeting the pertussis toxin promoter (ptxA-Pr) using hybridization probes. (a) Oligonucleotide primers and hybridization probes. Oligonucleotide primers were adapted from the work of Grimprel et al. (15) and manufactured by TibMolBiol (Berlin, Germany). A pair of fluorescence-labeled hybridization probes (ThermoBiol), PT2-FLU (5′-CTT CTA TCC TGG AGG GAT GAG ATT GAA CCG CAT GAA CGC TCC TTC-3′ phosphate group) and PT2-LCR640 (5′-LCred640-AATCCAACAAGGATGAA CGC TCC TTC TTC-3′ phosphate group) were designed and used for the real-time detection of the ptxA-Pr-specific PCR product. A third probe, ICPT-LCR (5′-LCred705-TGA CGT ACA TCA GCC TTC GAT GCT A-3′ phosphate group), was...
designed for the detection of the specific internal-control DNA constructed for this real-time PCR assay.

(b) Internal control. PCR inhibition was assessed by the addition of 4 fg of our specific internal control DNA (ICD-PT) to each sample tested, which was detected in duplex format. The strategy used to construct the internal control DNA (ICD) is the overlap extension technique (15), which allows construction of an ICD with the same primer binding sequences as the target DNA and containing a nontarget nucleic acid sequence (chimeric DNA). The constructed ICD is shorter (152 bp) than a natural PCR product and is inserted in the PCR4Blunt-TOPO plasmid (Invitrogen, California) to provide an unlimited amount of ICD. Furthermore, a specific probe (labeled with LCred705) could be designed as an ICD-PT containing chimeric DNA. The use of the same primers is an advantage, since multiple sets of primers might interfere with the amplification of one or both of the target genes.

The inclusion of 4 fg ICD-PT in the PCR mixture did not affect the sensitivity of the PCR for the detection of a ptx4-Pr-specific PCR product and allowed correct detection of PCR failure.

(c) ptx4-Pr PCR amplification. PCR amplification was performed using a real-time PCR system (LightCycler; Roche). The 20-μl reaction mixture contained 4 μl FastStart reaction mix hybridization probes (a component of the FastStart DNA MasterFlex/hybridization probe kit; Roche Diagnostics), 0.4 μl dimethyl sulfoxide, 1 μl Ucarin DNA glycosylase, 0.5 μM (each) primers PT1-Pr and PT2-Pr, 0.2 μM (each) probes PT2-LCR640 and ICPT-LCR, and 0.4 μM probe PT2-FLU.

Reaction conditions were 10 min at 95°C, followed by 50 cycles of 5 s at 95°C, 5 s at 60°C, and 8 s at 72°C. The fluorescence increase was measured during the annealing step at 66°C. A readout of LC-Red 640 values (B. pertussis-specific probe) was performed by using channel F2/back-F1 software, and a readout of LC-Red705 values (internal control DNA-specific product) was performed using channel F3/back-F2 software.

(d) Analytical sensitivity and specificity. A detection limit between 50 fg (equivalent to 10 CFU) and 5 fg (equivalent to 1 CFU) of B. pertussis template DNA was observed. No detection of the seven other Bordetella species, nor of non-Bordetella respiratory organisms, i.e., the Alcaligenaceae (Achromobacter spp., Alcaligenes spp., or Alcaligenes sp.) or Moraxellaceae (Brachmella spp. and Moraxella) families or Pasteurella spp. or Neisseria spp., was observed, confirming our previous data (13).

(ii) Detection of B. pertussis by real-time PCR targeting IS481 using hybridization probes. The real-time PCR using IS481 as the target was performed according to the recommendations of the PCR consensus meeting (24).

Serology. Serum samples were analyzed using ELISA to quantify IgG anti-PT antibody, as previously described (25), using purified PT from Sanofi Pasteur. The assay cutoff was set at 20 EU/ml. Positive paired serology was defined with antibody, as previously described (25), using purified PT from Sanofi Pasteur.

The sensitivity of each of the individual laboratory techniques including ptx4-Pr PCR, IS481 PCR, single serology with IgG antibody titers of ≥125 EU/ml, and paired serology with a fourfold change in IgG titers. For the calculation of the sensitivity of the diagnostic test i, true-positive cases were defined as symptomatic laboratory-confirmed cases with positive diagnostic test i and false-negative cases were defined as symptomatic laboratory-confirmed cases with a negative result on diagnostic test i. For the calculation of the specificity of a diagnostic test, true-negative cases were defined as epidemiologically linked pertussis cases, contacts with asymptomatic or subclinical pertussis infection, or contacts without evidence of recent pertussis infection with a negative diagnostic test i and false-positive cases as epidemiologically linked pertussis cases, contacts with asymptomatic or subclinical pertussis infection, or contacts without evidence of recent pertussis infection with a positive diagnostic test i.

We then determined the sensitivities and specificities of the combinations of different tests, including single and paired serology and PCR assays (ptx4-Pr or IS481) with single serology to estimate the ability to diagnosis pertussis using data collected at first presentation. Sensitivities and specificities were reported with 95% confidence intervals and compared using the Student t test. Key demographic and clinical characteristics of participants with and without missing laboratory data were compared using the chi-squared test. Laboratory diagnostics were ranked according to the Youden index (28), a summary measure of the reciprocal-operating-characteristics curve, to estimate the effectiveness of the diagnostic test. The Youden index was calculated as follows: [sensitivity (specificity) – 1]. A perfect diagnostic test, i.e., a test which allows complete separation of the diseased and healthy populations, has a Youden index of 1 (12).

Finally, we performed one sensitivity analysis, using a case definition with the other reported cutoff for single serology (IgG antibody titers of ≥100 EU/ml) and paired serology (twofold change in ELISA titers) with the same case and noncase definitions.

RESULTS

Description of the analyzed population. Among the 404 contacts included in the epidemiological study, 353 participants provided a serological sample at the inclusion visit. Within this group, 14 participants, of ages from 3 months to 2 years or 4 to 7 years, were excluded from the analysis. ptx4-Pr PCR and IS481 PCR were performed on samples from 289 and 288 participants, respectively. Complete laboratory data sets were available for 256 participants at the inclusion visit and 218 participants for both visits (Table 1). Compared with those with complete diagnostic data, participants with missing data were more likely to be male (52% versus 39%; P = 0.02), <18 years old (52.0% versus 30.7%; P < 0.0001), and symptomatic for pertussis (72.3% versus 60.1%; P = 0.03) (Table 2). Among the 218 contacts analyzed (of whom 195 were ≥7 years old, 21 were ≥3 months old and <7 years old, and 2 were <3 months old), 95 (of whom 82 were ≥7 years old) had a laboratory-confirmed symptomatic pertussis infection, 14 (of whom 10 were ≥7 years old) had a symptomatic epidemiologically linked pertussis infection, 33 (of whom 32 were ≥7 years old)
had an asymptomatic or subclinical pertussis infection, and 76 (of whom 71 were ≥7 years old) were totally free of pertussis infection.

**Analysis of isolates collected during the study.** Only 13 isolates were collected during the study. They were collected nine times for index cases and four times for mothers of index cases. These adults had all PCR- and serology-positive diagnoses. All isolates express the characterized toxins and adhesins. Genotyping of the PT S1 subunit showed that they all harbored similar ptxA1 and pm2 genes except for one U.S. isolate harboring a pm3 gene. We previously separated B. pertussis isolates into five major PFGE groups (6, 7, 25). All 13 isolates were included in the same PFGE group, group IV. All U.S. and French isolates were characterized as PFGE subgroup IV beta with one exception, which was PFGE subgroup IV gamma, and the three German ones were either PFGE subgroup alpha or beta.

**Comparative sensitivities and specificities of real-time PCR and serological assays for diagnosis of pertussis infection.**

(i) **Main analysis.** The number of positive tests by type of contact is presented in Table 3. The sensitivity and specificity of each diagnostic assay are presented for the 195 participants with complete data at both visits (Table 4).

Among PCR assays, the sensitivity of the *ptxA*-Pr PCR was significantly lower than the sensitivity of the IS481 PCR (15.9% versus 45.1%; *P* < 10⁻⁴), whereas the specificity of the *ptxA*-Pr PCR was significantly higher than that for IS481 PCR (97.3% versus 85.0%; *P* < 10⁻⁵).

Single serology was significantly more sensitive (64.6% versus 15.9%; *P* < 10⁻⁴) than paired serology and had comparable specificity (92.0% versus 89.4%; *P* < 0.19). Single serology was also more sensitive than the IS481 PCR (64.6% versus 45.1%; *P* < 10⁻⁴) and the *ptxA*-Pr PCR (64.6% versus 15.9%; *P* < 10⁻⁵).

Single serology was more specific than IS481 PCR (92.0% versus 85.0%; *P* < 0.02) but less specific than the *ptxA*-Pr PCR (92.0% versus 97.3%; *P* < 0.01).

Among all individual diagnostic assays, single serology was the most efficient assay, with Youden indices of 0.57, compared to <0.31 for all other assays.

As summarized by the Youden indices (Table 4), the most efficient laboratory diagnoses for symptomatic laboratory-confirmed pertussis cases were the combination of single serology with paired serology or one PCR test. The most sensitive test was the combination of single serology with IS481 PCR, and the most specific was the combination of single serology with *ptxA*-Pr PCR.

(ii) **Sensitivity analyses.** Using more-sensitive cutoffs for serology (≥100 and a twofold change), there were 84 cases and 111 noncases. Lowering the single-serum cutoff to ≥100 EU/ml or the cutoff for paired serology to a ≥2-fold change in titer slightly improved efficiency of individual tests due to a modest increase in sensitivity and a small decrease in specificity; nevertheless, the results for the estimated sensitivity and specificity of the laboratory tests were comparable to those reported in the main analysis (Table 5). The most specific diagnostic tests were the combination of single serology with *ptxA*-Pr PCR or IS481 PCR, and the most sensitive were the combination of single serology with paired serology or with the IS481 PCR.

**DISCUSSION**

This study was performed in order to compare different pertussis biological diagnostic techniques in current routine use. The source data were obtained from a multicenter household study on pertussis transmission (27) in which five diagnostic measures were used: culture, two PCR assays with different DNA targets, and single and paired PT ELISAs with two cutoffs.

Culture was not performed in all centers because of the difficulty in isolating *B. pertussis*, the lack of sensitivity, and the long delay necessary to obtain the result (8). However, it is important to note that pursuing culture is essential in order to...
analyze the spatio-temporal evolution of *Bordetella pertussis* according to the immunity of the human population. We observed that the collected isolates were very similar to each other (all PFGE group IV), confirming our previous results obtained in Argentina (4), Europe (7), Japan (14), Russia (17), and France (6, 26). We demonstrated that the PFGE group IV subgroup gamma, first observed in Finland and more recently in France (5, 7), is now also circulating in the United States.

Because of the long delay necessary to isolate the bacteria, many real-time PCR assays have been developed in recent years for routine diagnoses of pertussis. After a consensus meeting in 2005, recommendations were published (24). It was underlined that real-time *ptxA*-Pr PCR is less sensitive but more specific than IS481 PCR (24), since IS481 PCR can detect *Bordetella holmesii* species and some *Bordetella bronchiseptica* and *B. pertussis* spp. This may not impact substantially on test performance, since the incidence of *B. holmesii* respiratory infections seems to be very low, as is that of *B. bronchiseptica*, which infects mostly immunosuppressed individuals (2, 11). In the present study, we confirmed that *ptxA*-Pr real-time PCR is less sensitive than IS481 PCR and demonstrated that performing both *ptxA*-Pr and IS481 PCR does not improve diagnostic performance. Furthermore, we did not observe any *ptxA*-Pr PCR-positive case which was negative by IS481 PCR. This observation is in contrast with that of Qin et al. (23).

Qin et al. speculate that the underperformance of IS481 compared to that of the multiplex IS may have been a result of nonrandom genome degradation of IS481, which is considered a nonessential genomic element, during the course of host-pathogen interaction at a later disease stage. The difference between the two studies may also be explained by a difference in the study population, with the Qin study including mainly young children (median age, 2.5 years) suspected of having pertussis infection, whereas our study included contacts of infant index cases (median age, 27 years) investigated at a later stage of disease. However, even among those contacts that were assessed when symptomatic for a short period of time, we did not observe any *ptxA*-Pr PCR-positive case which was negative by IS481 PCR. Our data confirm that IS481 real-time PCR is a useful diagnostic tool.

ELISA determinations of anti-PT antibody titers are a validated and very sensitive diagnostic method which can be used with single serum or paired (acute- and convalescent-phase) serum samples (8). A fourfold change in the titers of anti-PT antibodies was believed to be the most sensitive and specific biological diagnosis (25). However, the paired serum sample assay is impractical for routine diagnosis. For this reason, single-sample serology has been developed and IgG cutoff values have been determined for a number of laboratories (3, 8, 9), and this method is now used for routine diagnosis. In the present study, we compared single and paired serology and two different cutoffs. Twofold changes were more sensitive but less specific than fourfold changes. Single serology, independently of the cutoff used, was more sensitive and more specific than paired serology, and the agreement was poor between single and paired serology results. We demonstrated that the perfor-

### TABLE 4. Comparative sensitivities, specificities, and Youden index for diagnosis of symptomatic laboratory-confirmed pertussis

<table>
<thead>
<tr>
<th>Method of diagnosis (cutoff)</th>
<th>% Sensitivity (95% CI)</th>
<th>% Specificity (95% CI)</th>
<th>Youden index</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ptxA</em>-Pr PCR</td>
<td>15.9 (10.8–21.0)</td>
<td>97.3 (95.1–99.5)</td>
<td>0.13</td>
</tr>
<tr>
<td>IS481 PCR</td>
<td>45.1 (38.1–52.1)</td>
<td>85.0 (80.0–90.0)</td>
<td>0.30</td>
</tr>
<tr>
<td>Single serology (≥125 EU/ml)</td>
<td>64.6 (57.9–71.3)</td>
<td>92.0 (88.2–95.8)</td>
<td>0.57</td>
</tr>
<tr>
<td>Paired serology (×4)</td>
<td>15.9 (10.8–21.0)</td>
<td>89.4 (85.1–93.7)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

Combination of single serology (≥125 EU/ml) with:

| Paired serology, (×4)       | 80.4 (74.8–86.0)       | 89.4 (85.1–93.7)        | 0.70         |
| *ptxA*-Pr PCR               | 75.6 (69.6–81.6)       | 98.2 (96.3–100)         | 0.74         |
| IS481 PCR                   | 89.0 (84.6–93.4)       | 79.6 (73.9–85.3)        | 0.69         |

* Subjects are contacts of infant pertussis cases for whom there is complete laboratory data (n = 195). Complete data include symptoms, serology, and PCR (IS481 and *ptx*-Pr) at enrollment and symptoms and serology at follow-up visit 1 month later.

### TABLE 5. Comparative sensitivities, specificities, and Youden index for diagnosis of symptomatic laboratory-confirmed pertussis

<table>
<thead>
<tr>
<th>Method of diagnosis (cutoff)</th>
<th>% Sensitivity (95% CI)</th>
<th>% Specificity (95% CI)</th>
<th>Youden index</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>ptxA</em>-Pr PCR</td>
<td>15.5 (10.4–20.6)</td>
<td>97.3 (95.0–99.6)</td>
<td>0.13</td>
</tr>
<tr>
<td>IS481 PCR</td>
<td>44.0 (37.0–51.0)</td>
<td>84.7 (79.6–89.8)</td>
<td>0.29</td>
</tr>
<tr>
<td>Single serology (≥100)</td>
<td>67.9 (61.3–74.5)</td>
<td>90.1 (85.9–94.3)</td>
<td>0.58</td>
</tr>
<tr>
<td>Paired serology (×2)</td>
<td>23.8 (17.8–29.8)</td>
<td>83.8 (78.6–89.0)</td>
<td>0.08</td>
</tr>
</tbody>
</table>

Combination of single serology (≥100 EU/ml) with:

| Paired serology (×2)        | 85.7 (80.8–90.6)       | 75.7 (69.7–81.7)        | 0.61         |
| *ptxA*-Pr PCR               | 77.4 (71.5–83.3)       | 87.4 (82.7–92.1)        | 0.65         |
| IS481 PCR                   | 90.5 (86.4–94.6)       | 77.5 (71.6–83.4)        | 0.68         |

* Subjects are contacts of infant pertussis cases for whom there is complete laboratory data (n = 195). Complete data include symptoms, serology, and PCR analysis (IS481 and *ptx*-Pr) at enrollment and symptoms and serology at follow-up visit 1 month later.

b ×2, twofold change in titer.

c CI, confidence interval.
mance of the combination of single serology with paired serology did not improve the performance of single serology and confirmed the usefulness of single serology for routine diagnosis of pertussis.

This study is subject to some limitations. Culture has traditionally served as an imperfect gold standard, but its poor sensitivity limits the ability to assess the performance of other diagnostic tests. Hence, an algorithm was developed which incorporated diagnostic information from all available sources (i.e., PCR, serum, and symptoms). While the use of an algorithm as a pooled gold standard is superior to treating a single imperfect diagnostic test as an absolute gold standard, using a pooled gold standard potentially results in overestimating the sensitivities and specificities of the individual tests included in the pooled gold standard (21). To compare the values of the different diagnostic tests, we presented the sensitivity, specificity, and Youden index for each of the tests assessed. The Youden index is an attractive single value for the effectiveness of a diagnostic marker in distinguishing between diseased and nondiseased populations. A limitation of the Youden index stems from the fact that it gives equal weight to the sensitivity and specificity. Depending on the setting in which the test is used, a health care provider or researcher may want to give greater weight to either the sensitivity or specificity of a particular diagnostic test.

All the participants included in these analyses were close contacts of confirmed cases of pertussis infection who had a higher probability of being exposed to *B. pertussis* than the general population and therefore had a higher probability of having a positive laboratory test.

In addition, this study design could have produced a relatively large number of asymptomatic contacts with positive laboratory tests, thus biasing the estimate of the sensitivity and specificity of these laboratory tests compared to those for the general population. Further research is needed to determine if the same levels of sensitivity and specificity and the same ranking of test efficacy are obtained with the general population.

In conclusion, IS481 PCR and single-serum serology are currently the most efficient diagnostic tools available for pertussis diagnosis for children of ages ≥7 years, adolescents, and adults in contact with a pertussis case. The combination of both assays provides the most sensitive diagnostic tool for pertussis at the time of presentation to a health care facility. Culture will remain valuable since it allows for the analysis of the spatio-temporal evolution of the bacterium.

The efficiency of single serology may require future reevaluation, however, because of changes in pertussis vaccines (replacement of whole-cell vaccines with acellular ones) and new vaccine recommendations which promote booster vaccinations for adolescents and adults (1, 18). Acellular vaccines contain fewer antigens than whole-cell vaccines and induce different and larger humoral immune responses than whole-cell vaccines (10). The use of single serum may thus decrease with more-widespread implementation of booster vaccinations, since serological diagnoses can be performed only after a waiting period of 2 to 3 years following a booster immunization with a pertussis acellular vaccine (19).

**Acknowledgments**

We thank Institut Pasteur Fondation, URA-CNRS 3012, and Sanofi-Pasteur for supporting this work financially.

**References**


