Comparison of culture and PCR for detection of \textit{Bordetella pertussis} and \textit{Bordetella parapertussis} under routine laboratory conditions

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A PCR assay for the detection of \textit{Bordetella pertussis} and \textit{Bordetella parapertussis} was compared with the conventional culture method under routine laboratory conditions. Detection of \textit{B. pertussis} was based on the amplification of a section of the IS481 insertion sequence and confirmation of positive results was based on a sequence of the pertussis toxin promoter region. Detection of \textit{B. parapertussis} was based on the amplification of a section of the IS1001 insertion sequence. An internal control was included. Data were available for the period 28 November 2000 to 9 July 2003. In this period, 3096 patients were examined for infection with \textit{B. pertussis} and \textit{B. parapertussis} by culture and PCR on the same day. \textit{B. pertussis} was found in 496 (16 \%) patients; 208 (42 \%) were diagnosed by PCR alone whereas 17 (3 \%) were diagnosed by culture alone. \textit{B. parapertussis} was found in 64 (2 \%) patients. The sensitivity of the PCR was 97 \% and of culture 58 \%. The specificity of PCR was 93 \% when regarding culture as 100 \% sensitive. There was a significant relationship between laboratory method and age, as the superiority of PCR was most marked in the age group 0.5–3 years. The PCR assay proved highly sensitive for the diagnosis of pertussis. The specificity estimate of the PCR assay suffers from the influence of a gold-standard method with a low sensitivity. The PCR assay is considered highly specific due to the amplification of two different sequences in two separate assays.

INTRODUCTION

Culture is the gold standard for diagnosis of infection with \textit{Bordetella pertussis}. The method is highly specific but the sensitivity is low, declining with the duration of illness and with the age of the patients (Cimolai \textit{et al}., 1996; Grimprel \textit{et al}., 1993; Wirsing von König \textit{et al}., 1990). Furthermore, culture is slow, as the charcoal agar plates need to be incubated for 4–5 days for \textit{B. pertussis} to grow. These disadvantages have increased the interest in more rapid methods for \textit{B. pertussis} diagnosis and more than 100 PCR protocols have been published since the late 1980s. In most comparisons of culture and PCR, the diagnostic yield has been increased about two- to fourfold by the use of PCR despite varying sampling methods, sample preparation methods, primers and detection methods (Heininger \textit{et al}., 2000; Loeffhelholz \textit{et al}., 1999; Schlaper \textit{et al}., 1995; Schmidt-Schlaper \textit{et al}., 1997; Tilley \textit{et al}., 2000; van der Zee \textit{et al}., 1996). Previously published PCR methods amplify a single gene sequence, most often within the insertion sequence IS481 (Aoyama \textit{et al}., 1997; Backman \textit{et al}., 1994; Glare \textit{et al}., 1990; Grimprel \textit{et al}., 1993; He \textit{et al}., 1993; Heininger \textit{et al}., 2000; Lichtinghagen \textit{et al}., 1994; Mastrantonio \textit{et al}., 1996; Reizenstein \textit{et al}., 1993; Schlaper \textit{et al}., 1995), which is found in about 80 copies in the genome of \textit{B. pertussis} (van der Zee \textit{et al}., 1993a), but which is also found in the genome of \textit{Bordetella holmesii} (Reischl \textit{et al}., 2001). Other target genes are the pertussis toxin (PT) promoter region (Grimprel \textit{et al}., 1993; Heininger \textit{et al}., 2000; Mastrantonio \textit{et al}., 1996; Reizenstein \textit{et al}., 1993; Schlaper \textit{et al}., 1995), the adenylate cyclase gene (Douglas \textit{et al}., 1993) and the porin gene (Farrell \textit{et al}., 2000; Li \textit{et al}., 1994). The challenge when comparing culture and PCR is to substantiate that the difference in performance of the two methods is due to the low sensitivity of culture rather than to a low specificity of the PCR method. A PCR method was implemented in our laboratory as a routine diagnostic method in 1998, with the intention of improving on the sensitivity of culture while retaining specificity. The use of more than one target for pertussis diagnosis by PCR in order to ensure high specificity was proposed by Qin \textit{et al}., (2002). The present study is an application of this recommendation under routine diagnostic conditions. The PCR method consisted of a screening assay amplifying IS481 (Glare \textit{et al}., 1990) and a confirmatory assay amplifying a sequence of the PT promoter region (Birkebæk \textit{et al}., 1994). Positive results of both tests should be

Abbreviations: IPC, internal process control; PT, pertussis toxin.
obtained to consider a specimen positive for *B. pertussis*. The two targets were detected in two separate assays in order to diminish further the risk of false-positive results due to sample-to-sample contamination.

In Denmark, more than 95 % of all specimens are analysed by our laboratory. Serological testing is not performed in Denmark, and has therefore not been included as a diagnostic criterion in the current study. Diagnosis by both culture and PCR was performed under routine conditions between 28 November 2000 and 9 July 2003.

**METHODS**

**Patients and specimens.** Specimens were obtained from patients at self-referral to a physician within the study period. The majority of the specimens were taken by peroral nasopharyngeal swabbing with a bent, charcoal-impregnated cotton wool swab. The swabs were transported in Stuart’s transport medium (Roeder, 1993) to the laboratory. Data were analysed from all patients who had a specimen taken for both culture and PCR results as well, only the first pair of culture and PCR results was used in the analysis.

**Culture.** On receipt, all swab specimens were inoculated on pertussis charcoal agar plates containing 23 mg cephalaxin 1 L and 10 % defibrinated horse blood (Roeder, 1993). Half of the plate was inoculated with the swab and spread further with a sterile loop as described previously (Kristensen, 1961). The charcoal plates were incubated at 36 °C in air and inspected with a stereomicroscope on days 4 and 5. Colonies were identified as *B. pertussis* or *B. parapertussis* by a direct immunofluorescence assay with specific antisera (Difco).

**PCR**

**Sample preparation.** Sample preparation of the swab material for PCR analysis was performed after inoculation on charcoal plates. Swabs were transferred to a 1·5 ml Eppendorf tube and extracted in 300 μl of 2SP chlamydia transport medium (SSI; Jensen et al., 1991). Samples (100 μl) were mixed with 300 μl 20 % Chelex in TE buffer, vortexed for 60 s and incubated at 94 °C for 10 min. The tube was centrifuged at 30 000 g for 5 min and 10 μl supernatant was used for the PCR (Jensen et al., 2003).

**Primers.** The IS481 primers originally described by Glare et al. (1990), designated BP1 (5′-GATCTAATAGGTGTAGATCATGGTT-3′) and a modification of BP2, BP2mod (5′-CAGCTTGGAGTTCTGC-3′) (bases in italics were designated BP1, BP2mod, BPPA and BPPZ, 2 μl of PlatinumTaq DNA (Invitrogen), 0·01 % BSA and an appropriate amount of the IPC corresponding to approximately 10–50 DNA copies. The *Taq* polymerase was activated by an initial incubation at 94 °C for 2 min followed by thermocycling in a Perkin Elmer 9600 thermocycler using a ‘touch-down’ procedure during the first 10 cycles, consisting of denaturation at 94 °C for 15 s, annealing at 66–56 °C for 30 s with a 1 °C decrement per cycle and extension at 72 °C for 30 s. Cycles 11–40 consisted of denaturation at 94 °C for 15 s, annealing at 56 °C for 15 s and extension at 72 °C for 30 s. After the last cycle, an extension step at 72 °C for 5 min was included.

**Screening assay.** Reactions were performed in 0·2 ml reaction tubes in a final volume of 100 μl containing 10X Platinum buffer (Invitrogen), 125 mM each of dATP, dGTP and dCTP, 250 μM dUTP, 3·5 mM MgCl2, 0·4 μM each primer BP1, BP2mod, BPPA and BPPZ, 2 μl of PlatinumTaq DNA (Invitrogen), 0·01 % BSA and an appropriate amount of the IPC corresponding to approximately 10–50 DNA copies. The *Taq* polymerase was activated by an initial incubation at 94 °C for 2 min followed by thermocycling in a Perkin Elmer 9600 thermocycler using a ‘touch-down’ procedure during the first 10 cycles, consisting of denaturation at 94 °C for 15 s, annealing at 66–56 °C for 30 s with a 1 °C decrement per cycle and extension at 72 °C for 30 s. Cycles 11–40 consisted of denaturation at 94 °C for 15 s, annealing at 56 °C for 15 s and extension at 72 °C for 30 s. After the last cycle, an extension step at 72 °C for 5 min was included.

**Confirmatory assay.** All *B. parapertussis*-positive test results were confirmed by repetition of the test with the same primers after preparation of a new sample, while all positive *B. pertussis* test results were confirmed using primers BPTOX F and BPTOX R. The reaction conditions were as for the screening assay except that Super *Taq* buffer (HT biotechnology) was used instead of Platinum buffer, giving 1·5 mM MgCl2 (final), and that the IPC was not used. Glycerol (5%) was added to avoid unspecific bands. The *Taq* polymerase was activated as described above. The ‘touch-down’ procedure consisted of 10 cycles of denaturation at 94 °C for 15 s, annealing at 72–62 °C for 30 s with a 1 °C decrement per cycle. Cycles 11–50 consisted of denaturation at 92 °C for 15 s, annealing at 62 °C for 30 s and extension at 72 °C for 30 s. After the last cycle an extension step at 72 °C for 5 min was included. If the screening assay gave rise to a faint band only, the confirmatory assay was performed with 10 and 20 μl of the template.

After PCR, the amplified samples were subjected to agarose gel electrophoresis and ethidium bromide staining.

**Controls.** Two positive and two negative controls were incorporated in each test. The positive controls consisted of two tenfold dilutions of a suspension of culture of the relevant species containing approximately 30 and 5 genome copies, respectively. If the higher copy number control was negative, the experiment was repeated. The more dilute control generated a faint band in the agarose gel in most of the experiments, serving as a control for a satisfactory detection limit. The negative controls consisted of sterile Milli-Q water and Milli-Q water subjected to sample preparation along with the clinical specimens. If one of the negative controls was positive, the analysis was repeated. If the IPC did not result in a fragment of the expected size in the negative samples, the analysis of the sample was repeated using 5 and 2 μl of template material.

**Procedures to diminish the risk of contamination.** Only DNase-free, sterile pipette tips with a filter barrier were used for the sample preparation and PCR set-up. Handling of the specimens before amplification was performed in laminar flow cabinets. Separate laboratories were used for culture and PCR set-up. The PCR set-up laboratory was situated in a building separate from the building performing the post-PCR analysis. Separate teams of technicians performed the culture, PCR set-up and post-PCR procedures.

**Statistics.** For both analyses, a logistic regression model was used. The

GACCTTGTAGTgacgtgattc-3′, and IPC BPPZ, 5′-CAGCGCTCTACGAGTTGGAGATgacatacggaaatag-3′ (sequences in lower-case letters correspond to the lambda sequence).

PCR products containing the binding sites of the specific primers were obtained by amplification of lambda DNA with an annealing temperature of 40 °C. After gel-purification of the amplicons, a tenfold titration was performed to find the dilution of the IPC producing no increase in the detection limit of the positive control (Jensen et al., 2003).

Internal process control (IPC). In order to detect the presence of *Taq* DNA polymerase inhibitors or sub-optimal reaction conditions an IPC was constructed. In brief, primers amplifying a part of the phage lambda genome were synthesized with a tail containing the sequence of each of the *B. parapertussis*-specific primers added to the 5′ end of the corresponding lambda primer; IPC BPPA, 5′-CGCGCTTGATGAGTTGGAGAT-3′, and IPC BPPZ, 5′-CAGCGCTCTACGAGTTGGAGAT-3′ (sequences in lower-case letters correspond to the lambda sequence).

Statistics. For both analyses, a logistic regression model was used. The
binary outcome corresponding to the diagnosis was used as the dependent variable. The independent variables included in the model were laboratory method (culture or PCR), age (grouped as 0–< 0.5, 0.5–3, 4–5, 6–9, 10–19 and 20+ years), gender and the date of collection of the specimen, grouped into four quarters, in order to control for seasonal variations in prevalence. In the model, first-order interactions between method and the remaining three independent variables were also included. Furthermore, the model also took into account the possible correlation between the results of culture and PCR originating from the same patient, using the GEE-estimation method (SAS 8.2, PROC GENMOD).

All estimates of unknown parameters are given with a 95 % confidence interval in parentheses.

RESULTS AND DISCUSSION

The laboratory results of 3096 patients were included in the analysis. The frequencies of the four possible combinations of outcomes of the two laboratory methods are shown in Table 1. Of the 496 patients (16 %) with a diagnosis of pertussis, based on a positive culture and/or PCR result, 208 (42 %) were positive by PCR only and 17 (3 %) were positive by culture only.

The sensitivity of culture was 58 % (288/496) and of PCR was 97 % (479/496) when considering both positive culture and PCR results as true positives. The specificity of the PCR was 93 % (2600/2808) if culture alone is considered to be the gold standard with a sensitivity of 100 %. The prevalence of positive findings with the two laboratory methods in the different age groups is given in Table 2. The prevalence was highest in the age group 6–9 years (24 % by PCR and 16 % by culture) and lowest in the age group 0.5–3 years (10 % by PCR and 4 % by culture).

There was a significant relationship between laboratory method and age ($P = 0.0003$); the superiority of PCR over culture was most marked in the age group 0.5–3 years [odds-ratio $= 3.16 (2.34–4.26)$] (Fig. 1). No significant relationship was found between laboratory method and gender ($P = 0.79$) or between laboratory method and season ($P = 0.41$). There was a borderline significant difference in the prevalence according to gender ($P = 0.0497$), female patients being more likely to be $B. pertussis$ positive [odds-ratio (female versus male patients) $= 1.23 (0.99–1.51)$]. There was a significant seasonal variation in prevalence ($P < 0.0001$), being highest in the third quarter (30 % by PCR and 19 % by culture).

Table 3 shows the results for $B. parapertussis$ diagnosis in the period. The overall prevalence of $B. parapertussis$ (culture-and/or PCR-confirmed) was 2 % (64/3096). The sensitivity

<table>
<thead>
<tr>
<th>Method</th>
<th>Age (years)</th>
<th>0–&lt; 0.5</th>
<th>0.5–3</th>
<th>4–5</th>
<th>6–9</th>
<th>10–19</th>
<th>20+</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td></td>
<td>50/356 (14)</td>
<td>36/1016 (4)</td>
<td>29/347 (8)</td>
<td>93/584 (16)</td>
<td>31/276 (11)</td>
<td>49/517 (9)</td>
<td>288/3096 (9)</td>
</tr>
<tr>
<td>PCR</td>
<td></td>
<td>75/356 (21)</td>
<td>98/1016 (10)</td>
<td>54/347 (16)</td>
<td>141/584 (24)</td>
<td>40/276 (15)</td>
<td>71/517 (14)</td>
<td>479/3096 (15)</td>
</tr>
</tbody>
</table>

Table 1. $B. pertussis$, culture and PCR results from 3096 patients, with at least one pair of a PCR and culture specimen taken on the same day

Values in parentheses represent percentages.

<table>
<thead>
<tr>
<th>B. pertussis</th>
<th>PCR-positive</th>
<th>PCR-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture-positive</td>
<td>271 (9)</td>
<td>17 (0.6)</td>
</tr>
<tr>
<td>Culture-negative</td>
<td>208 (7)</td>
<td>2600 (84)</td>
</tr>
</tbody>
</table>

Table 2. $B. pertussis$, culture and PCR results in age groups

Number of positives/total positives in age group; prevalence is given as percentages in parentheses.

<table>
<thead>
<tr>
<th>B. parapertussis</th>
<th>PCR-positive</th>
<th>PCR-negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture-positive</td>
<td>32 (1)</td>
<td>3 (0.1)</td>
</tr>
<tr>
<td>Culture-negative</td>
<td>29 (1)</td>
<td>3032 (98)</td>
</tr>
</tbody>
</table>

Table 3. $B. parapertussis$, culture and PCR results from 3096 patients with at least one pair of a PCR and culture specimen taken on the same day

Values in parentheses represent percentages.
of culture and PCR calculated as for pertussis was 55 % (35/64) and 95 % (61/64), respectively, and the specificity of PCR when the sensitivity of culture was considered 100 % was 99 % (3032/3061). Co-infection with *B. pertussis* and *B. parapertussis* was seen in five cases, all diagnosed by PCR.

In this study, we used data collected during routine laboratory diagnostic work to compare culture and PCR for pertussis diagnosis. The main advantages of this approach were that we had a large dataset, collected under routine diagnostic conditions. Thus, the results could be considered indicative of the true assay performance since they were not biased due to study protocols using selected research technicians. The disadvantages were that no clinical data were available. In addition, it was not possible to estimate the performance of the screening assay and the confirmatory assay separately, as only a conclusive result of the two assays together was available.

In Denmark, peroral nasopharyngeal swabbing is the recommended method of specimen collection for *B. pertussis* diagnostics. This method has proved at least as effective as pernasal nasopharyngeal swab collection for culture and PCR in a clinical trial recently carried out in Denmark (D. Dragsted, L. Berthelsen, J. Madsen and S. Hoffmann, unpublished results). We are convinced that the use of peroral swab collection did not reduce the performance of either of the laboratory methods used.

The main factors that determine the quality of the PCR method are the primers, which should target a highly specific and preserved sequence in the bacterial genome and the presence of sufficient laboratory facilities. This includes the use of adequate equipment and skilled personnel in order to minimize the risk of contamination of patient specimens with amplified bacterial DNA or directly from other clinical specimens, giving rise to false positive results.

Because of the high degree of genetic similarity between the different *Bordetella* species infecting the respiratory tract of humans (Arico & Rappuoli, 1987; van der Zee et al., 1997), selection of species-specific genetic sequences for pertussis diagnosis that at the same time are conserved within the species has been a major achievement. IS481, which is found in 80–100 copies (van der Zee et al., 1993a) in the *B. pertussis* genome, was described in 1988 and was found to be specific to *B. pertussis* relative to *B. parapertussis* and *Bordetella bronchiseptica* (McLafferty et al., 1988). Recently, however, it has been found that *B. holmesii*, described in 1995, also harbours the IS481 element (Reischl et al., 2001). *B. holmesii* has mainly been associated with septicemia in immunocompromised patients (Shepard et al., 2004), but has also been reported to give rise to pertussis-like symptoms, being responsible for 3.5 % of *Bordetella* respiratory infections (Yih et al., 1999; Mazengia et al., 2000).

The entire PT gene shows a high degree of similarity between different *Bordetella* species. The PT promoter region, however, shows polymorphisms due to point mutations responsible for the PT being produced by *B. pertussis* only (Arico & Rappuoli, 1987). In Table 4, the specificity of the primer sets used is indicated, with respect to other *Bordetella* species (represented by the ATCC type strains). In agreement with the recommendations from Qin et al. (2002), we strongly recommend the use of more than one target for diagnostic *B. pertussis* PCR assays. Furthermore, continuous surveillance of circulating strains, with regard to genetic changes in the regions used for PCR, should be carried out, in order to recognize changes that may have implications for the diagnostic sensitivity.

The two targets used in our assay are by far the most commonly used. The PCR assay containing the IPC was designed with a known, limited quantity of the IPC in order to avoid competition with the specific targets, which could result in a loss in sensitivity of the PCR assay. Obviously, this approach does not control for the presence of human cells in the specimen, but using a human gene to control for inhibition would not detect partial inhibition due to their presence in high copy numbers. Furthermore, the limit of detection for the specific targets is likely to increase.

In order to compare the two laboratory methods on a patient basis, we supplemented the analysis including results of patients who had specimens taken for culture and PCR on the same day, with an analysis of data from patients having at least one PCR specimen and at least one culture specimen submitted within 7 days. In this analysis, the patient was considered culture-positive if at least one culture specimen was positive, and PCR-positive if at least one PCR specimen was positive. It was expected that more of the PCR-positive results would have been culture confirmed, as it is known that the sensitivity of one specimen is lower than that of two

Table 4. Specificity of the primer sets used in the PCR assay with respect to different *Bordetella* species

<table>
<thead>
<tr>
<th>Species</th>
<th>IS481 primers</th>
<th>PT primers</th>
<th>IS1001 primers</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. pertussis</em> (ATCC 9797T)</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td><em>B. parapertussis</em> (ATCC 15311T)</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td><em>B. bronchiseptica</em> (ATCC 19395T)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>B. holmesii</em> (ATCC 51541T)</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Bordetella hinzii</em> (ATCC 51783T)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>Bordetella avium</em> (ATCC 35086T)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
or three specimens for culture (Faber et al., 1997). The results of the two analyses proved to be very similar, probably because most patients had only one specimen taken which was sent for culture and PCR. Thus, only a few extra culture and PCR results were included in the analysis on a patient basis.

We found a much more marked difference in the performance of the two methods in the age group 0.5–3 years than in any other age group. This age group covers children from the age where they are expected to have received two doses of the pertussis vaccine. Thus, they are defined as immunized and in their first post-vaccination years, where immunization is expected to be most effective. In good agreement with this, it was the age group with the overall lowest prevalence of pertussis. The superiority of the PCR in this age group could be explained by the effect of vaccination leading to a lower bacterial load in these children, not sufficient to be detected by culture. Alternatively, the bacteria, though able to cause symptoms, are too attenuated to survive transport to the laboratory. To clarify this issue, the quantitative PCR assays described by several groups (Farrell et al., 2000; Templeton et al., 2003) could be of use. Previous reports have not been conclusive concerning the relationship between age and superior detection by PCR in relation to culture. Some authors have claimed it independent of age, despite results very similar to ours (van der Zee et al., 1996). In contrast, others have reported the increased detection of pertussis cases by PCR among adults (Cimolai et al., 1996). To some extent these differences might be explained by the different stratification according to age used in the studies.

We defined the age groups on the basis of changes in prevalence according to age in the study population. Based on a different age classification, and therefore not directly comparable, Tilley et al. (2000) found a high prevalence of pertussis in children less than 2 years of age and in adolescents, in comparison to the prevalence in children aged 2–10 years and in adults. They found no difference in sensitivity for any diagnostic method according to age group (Tilley et al., 2000).

A slight difference in the prevalence of pertussis according to gender has previously been reported for culture-confirmed cases (Farizo et al., 1992; Muller et al., 1986). A number of factors could influence this finding, such as differences in the rate of specimen collection and the use of empirical (and perhaps efficient) antibiotic treatment. As might well be expected, there was no relationship to the laboratory methods used. The finding of a seasonal variation in the prevalence of pertussis, which has also previously been reported for culture-confirmed cases (Christie et al., 1994; Farizo et al., 1992; Fine & Clarkson, 1986), was not related to the laboratory method used. This indicates that the seasonal variation was not due to the pertussis bacteria surviving better during transport at certain times of the year.

In conclusion, we found the PCR method applied in our laboratory to be both sensitive and specific when compared to culture. We are convinced that the specificity of the PCR method is much higher than that calculated using culture as the gold standard. Although our PCR method is performed with two separate primer sets in two separate assays, we find the loss of sensitivity caused by this procedure is limited and far outweighed by the advantages regarding specificity. In addition, the inclusion of an IPC significantly increased the reliability of the results. The finding that PCR is markedly more sensitive in the age group 0.5–3 years is important in relation to protection of unvaccinated infants, as older siblings are the most frequently reported source of infection of infants in Denmark (Faber et al., 1997). Considering the higher sensitivity and earlier availability of a positive PCR test result, prevention strategies can be significantly improved with fewer serious infections among highly susceptible infants as a result.

REFERENCES


He, Q., Mertsola, J., Soini, H., Skurnik, M., Ruuskanen, O. & Viljanen,


