Real-time PCR-based detection of *Bordetella pertussis* and *Bordetella parapertussis* in an Irish paediatric population

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Novel real-time PCR assays targeting the *Bordetella pertussis* insertion sequence IS₄₈₁, the toxin promoter region and *Bordetella parapertussis* insertion sequence IS₁₀₀₁ were designed. PCR assays were capable of detecting ≤10 copies of target DNA per reaction, with an amplification efficiency of ≥90 %. From September 2003 to December 2009, per-nasal swabs and nasopharyngeal aspirates submitted for *B. pertussis* culture from patients ≤1 month to >15 years of age were examined by real-time PCR. Among 1324 patients, 76 (5.7 %) were *B. pertussis* culture positive and 145 (10.95 %) were *B. pertussis* PCR positive. Of the *B. pertussis* PCR-positive patients, 117 (81 %) were aged 6 months or less. A total of 1548 samples were examined, of which 87 (5.6 %) were culture positive for *B. pertussis* and 169 (10.92 %) were *B. pertussis* PCR positive. All culture-positive samples were PCR positive. Seven specimens (0.5 %) were *B. parapertussis* culture positive and 10 (0.8 %) were *B. parapertussis* PCR positive, with all culture-positive samples yielding PCR-positive results. A review of patient laboratory records showed that of the 1324 patients tested for pertussis 555 (42 %) had samples referred for respiratory syncytial virus (RSV) testing and 165 (30 %) were positive, as compared to 19.4 % of the total 5719 patients tested for RSV in this period. Analysis of the age distribution of RSV-positive patients identified that 129 (78 %) were aged 6 months or less, similar to the incidence observed for pertussis in that patient age group. In conclusion, the introduction of the real-time PCR assays for the routine detection of *B. pertussis* resulted in a 91 % increase in the detection of the organism as compared to microbiological culture. The incidence of infection with *B. parapertussis* is low while the incidence of RSV infection in infants suspected of having pertussis is high, with a similar age distribution to *B. pertussis* infection.

**INTRODUCTION**

Our Lady’s Children’s Hospital in Dublin is the largest specialist paediatric hospital in the Republic of Ireland and provides secondary care locally and tertiary care nationally. Annually the Microbiology Laboratory receives in excess of 200 requests for *Bordetella pertussis* culture. The isolation of *B. pertussis* by culture is insensitive and is affected by a number of factors, including patient age, vaccination status, duration of illness, sample quality, swab type, transport medium and culture conditions (Mattoo & Cherry, 2005; Lievano et al., 2002).

Pertussis may also be diagnosed by the demonstration of a specific antibody response to pertussis toxin, where the demonstration of a single high value of IgG or IgA is diagnostic of a recent or active infection (André et al., 2008; de Melker et al., 2000). The use of a single 'high-titre' serum serology and PCR targeting the IS₄₈₁ insertion sequence are considered the most efficient diagnostic tools for pertussis in children >7 years, adolescents and adults (André et al., 2008). However, the use of serology for pertussis diagnosis in younger children is less useful due to recent vaccination, and the lack of pertussis toxin antibody response in up to 24 % of this age group (Stehr et al., 1998). Diagnostic serology cannot be validly interpreted for one year post-vaccination with acellular pertussis vaccines (Guiso et al., 2011).

It has been reported that pertussis is underdiagnosed in the Republic of Ireland due to the lack of diagnostic tests other than culture, so that diagnosis is based on symptoms (Shahdadpuri et al., 2004). The purpose of this study was...
to develop a real-time PCR assay for the detection of pertussis and to examine the age distribution of the disease in a paediatric population. The multi-copy IS481 is the most popular PCR target used to detect B. pertussis (Muyldermans et al., 2005). As the IS481 sequence occurs in Bordetella holmesii (Reischl et al., 2001) and also in some strains of B. bronchiseptica and B. parapertussis a confirmatory PCR using an alternative gene target is required for specific detection of the organism. B. parapertussis may be a cause of infection indistinguishable from pertussis, and a PCR assay targeting this organism was designed as part of this study.

Pertussis in infants frequently presents with apnoea (Mattoo & Cherry, 2005). Life-threatening apnoea may also occur in very young infants with viral respiratory infection. Respiratory syncytial virus (RSV) is the most common cause of viral lower respiratory tract infection in infants and young children (van Woensel et al., 2003). The difficulty of distinguishing between the two infections is confounded by the fact that the age distributions of RSV infection and B. pertussis infection are thought to be very similar. This study also aimed to examine the prevalence of RSV in patients who had clinical samples submitted to the laboratory for pertussis screening.

METHODS

Primer and probe design. DNA sequences from target genes were retrieved from GenBank (http://www.ncbi.nlm.nih.gov) and aligned using the CLUSTAL W (1.82) multiple sequence alignment packages (http://www.ebi.ac.uk). The GenBank accession numbers of the sequences used for B. pertussis IS481 real-time PCR assay design are M28220, M20301, L26973, S66937 and S66929. GenBank accession numbers AF15732–AF157344 were used for B. pertussis toxin promoter real-time PCR assay design. The GenBank accession numbers of B. bronchiseptica toxin promoter sequences were used for sequence comparison are AY568397–AY568409 and AF157362–AF157364. The GenBank accession numbers of B. parapertussis toxin promoter sequences retrieved for sequence comparisons are AF157348–AF157361. The GenBank accession number of the B. parapertussis IS1001 sequence used for real-time PCR assay design is X66858.

Sequence areas for primer and probe design were selected on the basis of intra-species homology and inter-species heterogeneity. Primer Express software (Version 2.0 Applied Biosystems) was used to design the primers and MGBNFQ (minor groove binder/non-fluorescent quencher) probes (Table 1). All primers and probes were synthesized by Applied Biosystems.

Preparation of bacterial genomic DNA. Genomic DNA of B. pertussis ATCC 8467, B. parapertussis ATCC 15311, B. holmesii ATCC 51541 and B. bronchiseptica ATCC 19395 was isolated using the QIAamp DNA mini kit (Qiagen) (without an RNase A step) according to the manufacturer’s instructions. DNA was quantified by spectrophotometry at 260 nm. The specificity and sensitivity of assays was examined using 10-fold serial dilutions (from 18,000 pg to 0.18 pg) of the bacterial genomic DNA samples. Genomic DNA was also used as template for the preparation of positive control plasmid DNA standards.

To further test the specificity of the assays genomic DNA was isolated from the following bacteria using the QIAGene DNA mini kit: Bordetella petrii NCTC 13317, B. bronchiseptica NCTC 10539, B. avium NCTC 12033, B. anisorpii NCTC 13364, B. pertussis NCTC 10739, B. pertussis NCTC 13251, B. trematod NCTC 13203, B. hinzii NCTC 13199, B. holmesii ATCC 51541, B. parapertussis NCTC 15311, Pseudomonas aeruginosa ATCC 27853, Escherichia coli ATCC 25922, E. coli ATCC 35218, Staphylococcus aureus ATCC 25923, Staph. aureus ATCC 43300, Staph. aureus ATCC 29213, Haemophilus influenzae ATCC 10211, H. influenzae (wild-type), Klebsiella pneumoniae ATCC 700603, Yersinia enterocolitica ATCC 27729, Proteus mirabilis ATCC 43071, P. mirabilis ATCC 12453, Streptococcus pyogenes ATCC 19615, Strep. agalactiae ATCC 27956, Strep. salivarius ATCC 13419, Strep. pneumoniae ATCC 6305, Moraxella catarrhalis ATCC 25238, Enterococcus faecalis ATCC 51299, Ent. gallinarum ATCC 700425, Ent. cassilflavus ATCC 700327, Corynebacterium pseudodiphthericum ATCC 10700, Bacteroides fragilis ATCC 25285, Clostridium perfringens ATCC 13124 and Fusobacterium necrophorum ATCC 25286. Extracted DNA was quantified spectrophotometrically and 2 ng of the genomic DNA per reaction well was used as template. All bacterial isolates were tested in duplicate wells in the B. pertussis IS481, B. pertussis toxin promoter and B. parapertussis IS1001 assays. To verify that all extracted DNA was amplifiable, the extracted genomic DNA from all bacterial isolates was tested for the presence of the 16S rDNA. Primers (50 nM) as described by Mohammadi et al. (2005) and a FAM-labelled MGB probe (CGTATACCCGGGCTTG, 100 nM) were used to target the 16S rDNA in duplicate real-time PCRs.

Preparation of plasmid DNA standards to determine PCR amplification efficiency and assay sensitivity. PCR primer sets targeting DNA sequences external to the real-time PCR target amplicons were designed (Table 2) and used to prepare positive-control plasmid DNA standards for each of the real-time PCR assays. Solution-phase PCRs in a final volume of 50 μl contained 15 mM Tris/HCl, pH 8.0, 50 mM KCl, 15 % glycerol, 0.8 μM external primers, 200 μM (each) dNTP, 2–4 mM MgCl2, 2.5 units AmpliTaq Gold (Applied Biosystems) and 60 ng template bacterial genomic DNA. Amplification reactions were performed on a Perkin Elmer GeneAmp PCR 2400 thermocycler. Cycling conditions were as follows: pre-denaturation/enzyme activation at 95 °C for 10 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s; extension at 72 °C for 1 min 30 s, with a final extension at 72 °C for 10 min. PCR products were cloned using the TOPO TA Cloning system (Invitrogen). Plasmid DNA was isolated using the QIAprep miniprep kit (Qiagen) according to the manufacturer’s instructions. The nucleotide base sequences of the plasmid DNA inserts were determined by nucleotide sequencing of both DNA strands (Lark Technologies). The sensitivity, amplification efficiency and linearity range of the real-time PCR assays developed were demonstrated by amplifying duplicate aliquots of 10-fold serial dilutions (10^(-10) copies) of the appropriate plasmid DNA standard.

Real-time PCR amplification. The primer and probe concentration for each assay was optimized and concentrations that gave the lowest Cq (threshold cycle) and the highest ΔΔCt (the normalized reporter signal minus the baseline signal established in the first few cycles of the PCR) were selected for routine use (Table 1). Reaction volumes of 25 μl contained 1× TaqMan Mastermix (containing AmpErase uracil-N-glycosylase, with dTTP partially replaced by dUTP), the appropriate forward and reverse primer and MGBNFQ probe at concentrations as detailed in Table 1, and 4 μl template DNA (plasmid dilution, genomic DNA or extracted clinical sample). Real-time PCRs were performed in duplicate on an ABI Prism 7000 or 7500 sequence detector (Applied Biosystems) using universal thermal cycling conditions: 2 min at 50 °C (uracil N-glycosylase step), 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C.

Controls to minimize false-negative or -positive results. To minimize the potential of false-negative or false-positive results
Extracted specimens were analysed in duplicate wells for the presence of the human actin gene using a pre-designed assay (Applied Biosystems). The detection of human DNA served as a quality check of negative extraction controls, no-template controls, and positive amplification controls (quantified plasmid DNA controls). The inclusion of uracil-DNA glycosylase decontamination as a pre-amplification step further reduced the possibility of cross-contamination.

Extracted specimens were analysed in duplicate wells for the presence of the human β-actin gene using a pre-designed assay (Applied Biosystems). The detection of human DNA served as a quality check of negative extraction controls, no-template controls, and positive amplification controls (quantified plasmid DNA controls). The inclusion of uracil-DNA glycosylase decontamination as a pre-amplification step further reduced the possibility of cross-contamination.

**Table 1. Real-time oligonucleotide primers and probes designed and used in this study**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′–3′)*</th>
<th>Position†</th>
<th>Concen (µM)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pertussis IS481 Fwd</td>
<td>CAAGGCCGAACGCTTCTAT</td>
<td>862–879</td>
<td>0.05</td>
</tr>
<tr>
<td>Pertussis IS481 Rev</td>
<td>AGTTCTTGTTAGTTGAGGCTTAA</td>
<td>903–926</td>
<td>0.05</td>
</tr>
<tr>
<td>Pertussis IS481 probe</td>
<td>6-FAM-CAGTCCGCTGCTGGCT-MGB</td>
<td>881–895</td>
<td>0.05</td>
</tr>
<tr>
<td>Pertussis toxin prom Fwd</td>
<td>TTGCTGTTACAAAAACCTCGA</td>
<td>60–81</td>
<td>0.60</td>
</tr>
<tr>
<td>Pertussis toxin prom Rev</td>
<td>GTTCATGCGGCTTTGGATGTG</td>
<td>104–123</td>
<td>0.60</td>
</tr>
<tr>
<td>Pertussis toxin prom probe</td>
<td>6-FAM-CTTCGTTACATGCT-MGB</td>
<td>83–96</td>
<td>0.20</td>
</tr>
<tr>
<td>Parap IS1001 Fwd</td>
<td>CCGGCTGACGAGATTCG</td>
<td>952–968</td>
<td>0.30</td>
</tr>
<tr>
<td>Parap IS1001 Rev</td>
<td>AGAACGCTAGCCGGTTGAGTT</td>
<td>1013–1034</td>
<td>0.30</td>
</tr>
<tr>
<td>Parap IS1001 probe</td>
<td>6-FAM-AGCCAACCCCGCCT-MGB</td>
<td>973–986</td>
<td>0.20</td>
</tr>
</tbody>
</table>

*Position of each primer or probe is relative to the numbering of the following gene sequences: accession number M28220 for pertussis IS481; accession number AF157344 for pertussis toxin promoter external reverse, accession number BX640422.1 for pertussis toxin promoter forward external, and accession number X66858 for parapertussis IS1001.
†Oligonucleotide concentration used real-time PCR amplification reactions.

(Olivano et al., 2002) a series of real-time PCR amplification controls were incorporated into the protocol for the routine screening of respiratory specimens. All PCR plates contained negative extraction controls, no-template controls, and positive amplification controls (quantified plasmid DNA controls). The inclusion of uracil-DNA glycosylase decontamination as a pre-amplification step further reduced the possibility of cross-contamination.

**Table 2. Oligonucleotide primers used for the preparation of positive-control DNA plasmid standards**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5′–3′)§</th>
<th>Position†</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pertussis IS481 Fwd ext</td>
<td>TCAAGGCCGAACGCTTCTAT</td>
<td>702–723</td>
<td>342</td>
</tr>
<tr>
<td>Pertussis IS481 Rev ext</td>
<td>GTTGTATTGCTCCAGGTGAG</td>
<td>1022–1043</td>
<td>218</td>
</tr>
<tr>
<td>Pertussis toxin prom Fwd ext</td>
<td>GAAACCGCCGCGCAACGCT</td>
<td>480–496</td>
<td>218</td>
</tr>
<tr>
<td>Pertussis toxin prom Rev ext</td>
<td>CGGACGGTACGCCGTACCAT</td>
<td>148–167</td>
<td>445</td>
</tr>
<tr>
<td>Parapertussis IS1001 Fwd ext</td>
<td>CGCCCAACCCCGCTTCT</td>
<td>697–711</td>
<td>445</td>
</tr>
<tr>
<td>Parapertussis IS1001 Rev ext</td>
<td>CGATTGTCCTCCAGGTCTTC</td>
<td>1055–1075</td>
<td>445</td>
</tr>
</tbody>
</table>

§Position of each primer or probe is relative to the numbering of the following gene sequences: accession number M28220 for pertussis IS481; accession number AF157344 for pertussis toxin promoter external reverse, accession number BX640422.1 for pertussis toxin promoter forward external, and accession number X66858 for parapertussis IS1001.
tested and the number positive for RSV in this period were also determined.

RESULTS

Real-time PCR sensitivity

Tenfold serial dilutions of plasmid DNA controls (from $10^6$ to $10^0$ copies) for each assay yielded standard curves (B. pertussis IS481, slope = $-3.58$ and $y$-intercept = $93.40$; B. pertussis toxin promoter, slope = $-3.57$ and $y$-intercept = $93.66$; B. parapertussis IS1001, slope = $-3.575$, $y$-intercept = $40.82$), with strong correlation coefficients ($r^2 \geq 0.99$), indicating strong linear relationships over the 7-log range examined. PCR amplification efficiency for each assay was calculated from the slope of the standard curves [% efficiency = $(10^{-1/slope} - 1) \times 100$] and ranged from 90% to 91%. A detection limit of $\leq 10$ copies of target DNA per reaction was determined for all PCR assays, indicating high assay sensitivity.

Specificity of PCR assay targeting B. pertussis IS481

Throat swabs taken from healthy volunteers were negative by real-time PCR targeting B. pertussis IS481. Genomic DNA from B. petrii NCTC 13317, B. bronchiseptica NCTC 10539 and ATCC 19395, B. avium NCTC 12033, B. anserpii NCTC 13364, B. trematum NCTC 13203, B. hinzii NCTC 13199, B. parapertussis ATCC 15311 and human genomic DNA (Applied Biosystems) showed no cross-reaction with the B. pertussis IS481 assay. All dilutions of B. pertussis genomic DNA (18 000 pg to 0.18 pg) were detected in the real-time PCR assay for B. pertussis IS481. As expected, B. holmesii was detected with the assay targeting B. pertussis IS481, albeit with much lower sensitivity, B. pertussis being detected up to six cycles earlier. None of the non-Bordetella species used to examine the specificity of the assay were detected by real-time PCR.

Specificity of PCR assay targeting the B. pertussis toxin promoter

Genomic DNA from B. holmesii ATCC 51541, B. petrii NCTC 13317, B. avium NCTC 12033, B. anserpii NCTC 13364, B. trematum NCTC 13203, B. hinzii NCTC 13199, B. parapertussis ATCC 15311 and human genomic DNA were not detected by real-time PCR targeting the B. pertussis toxin promoter. Throat swabs from healthy volunteers were also PCR negative for the gene target. All dilutions of B. pertussis genomic DNA (18 000 pg to 0.18 pg) were detected in the real-time PCR for B. pertussis toxin promoter PCR. When 2 ng genomic DNA was used as template in the B. pertussis toxin promoter assay the genomic DNA was detected 3–8 cycles later than with the IS481 assay, depending on the B. pertussis strain tested, the difference being greatest with B. pertussis NCTC 10739. None of the non-Bordetella species were detected with the assay.

Due to extensive sequence homology between the B. pertussis and B. bronchiseptica toxin promoter regions, primers and probes targeting the B. pertussis toxin promoter were designed to maximize the difference between the B. bronchiseptica and B. pertussis primer and probe annealing regions, with four base-pair differences in the forward primer, two base-pair differences in the probe and three base-pair differences in the reverse primer. A weak PCR amplification was observed with B. bronchiseptica genomic DNA as template, with an 8–11 cycle difference in the detection of equivalent dilutions of B. bronchiseptica genomic DNA as compared to B. pertussis genomic DNA. The $\Delta R_n$ observed with B. bronchiseptica genomic DNA as template was also significantly lower, indicating that the DNA of B. bronchiseptica was detected much less efficiently.

Specificity of PCR assay targeting B. parapertussis IS1001

Genomic DNA from B. petrii NCTC 13317, B. bronchiseptica NCTC 10539, B. bronchiseptica ATCC 19395, B. avium NCTC 12033, B. anserpii NCTC 13364, B. pertussis NCTC 10739, B. pertussis NCTC 13251, B. pertussis ATCC 8467, B. trematum NCTC 13203, B. hinzii NCTC 13199, B. holmesii ATCC 51541, human genomic DNA, and DNA extracted from throat swabs of 20 healthy volunteers were not detected in the real-time PCR assay targeting B. parapertussis IS1001. All dilutions of B. parapertussis genomic DNA (18 000 pg to 0.18 pg) were detected in the real-time PCR B. parapertussis assay targeting IS1001. None of the non-Bordetella species were detected with the B. parapertussis IS1001 assay.

Interpretation of patient sample PCR results

DNA extracted from all clinical samples was tested in duplicate wells for four PCR targets: $\beta$-actin human DNA, B. pertussis IS481, B. pertussis toxin promoter and B. parapertussis IS1001. Specimens were reported PCR positive for B. pertussis only when both B. pertussis PCR targets (IS481 and toxin promoter) were positive in both real-time PCR wells. A positive IS481 PCR ($C_t < 36$) and a negative toxin promoter PCR was considered to be evidence of the presence of B. holmesii. A positive IS481 PCR with a $C_t > 36$ and a negative toxin promoter PCR was considered indicative of the possible presence of B. holmesii or a weak positive reaction for B. pertussis as the IS481 assay is for a multi-copy gene and the toxin promoter is a single-copy gene. A positive reaction in the toxin promoter PCR only may be indicative of the presence of B. bronchiseptica. Specimens were reported positive for B. parapertussis when both duplicate PCR wells tested positive for the B. parapertussis IS1001 target by real-time PCR.

Patient sample results

In total 1548 samples from 1324 patients were tested for the presence of B. pertussis and B. parapertussis. A total of 145 B. pertussis PCR-positive patients and 169 B. pertussis
PCR-positive samples were identified. Six samples were observed to have a mean $C_t$ >36 in the *B. pertussis* IS481 assay only, with no observable amplification with the *B. pertussis* promoter assay, and were reported as equivocal. A single sample produced a real-time PCR-positive result in the *B. pertussis* promoter assay only, with no observable amplification with the IS481 assay. Consistent with the criteria determined for reporting *B. pertussis* PCR results, which require that both *B. pertussis* targets be positive in duplicate wells for a specimen to be considered PCR-positive for *B. pertussis*, this sample was reported as negative for *B. pertussis*. As the toxin promoter PCR may test positive for *B. bronchiseptica*, infection with this organism cannot be ruled out, although only normal flora was isolated in culture.

A comparison of the result of real-time PCR detection and microbiological culture identified that 48.5% (82/169) of PCR-positive samples and 47% (69/145) of PCR-positive patients were culture negative for *B. pertussis*. All culture-positive samples were positive by PCR. The age distribution of patients and of positive specimens is detailed in Table 3. Of the *B. pertussis* PCR-positive samples 29 were nasopharyngeal aspirates. Of these 29 PCR-positive nasopharyngeal samples only 10 were culture positive.

Among the 1324 patients and 1548 samples examined in this study, 7 specimens were culture positive for *B. parapertussis* while 10 specimens were positive for *B. parapertussis* by real-time PCR. All seven of the culture-positive samples were PCR positive for *B. parapertussis*. The three specimens that were negative by culture had $C_t$ values of >37 cycles, indicating the presence of very small quantities of the genomic DNA target in extracted clinical specimens. A total of 27 samples were reported as unsuitable as the $C_t$ for $\beta$-actin was >36. These samples also tested negative for the presence of *B. pertussis* and *B. parapertussis*.

### Table 3. Age distribution of pertussis detected by real-time PCR and culture

<table>
<thead>
<tr>
<th>Age</th>
<th>Total patients</th>
<th><em>B. pertussis</em></th>
<th><em>B. parapertussis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCR positive (%)</td>
<td>Culture positive (%)</td>
<td>PCR positive (%)</td>
</tr>
<tr>
<td>≤1 month</td>
<td>338</td>
<td>49 (14.5)</td>
<td>29 (8.5)</td>
</tr>
<tr>
<td>2–6 months</td>
<td>441</td>
<td>68 (15.4)</td>
<td>33 (7.5)</td>
</tr>
<tr>
<td>7 months–11 years</td>
<td>503</td>
<td>27 (5.4)</td>
<td>13 (2.6)</td>
</tr>
<tr>
<td>12–15 years</td>
<td>24</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>&gt;15 years</td>
<td>18</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1324</strong></td>
<td><strong>145 (10.95)</strong></td>
<td><strong>76 (5.7)</strong></td>
</tr>
</tbody>
</table>

### RSV results

Among 1324 patients tested for pertussis, 555 (42%) had samples referred to the National Virus Reference Laboratory for RSV testing and 165 (30%) of those tested were positive. Analysis of the age distribution of RSV-positive patients showed that 129 (78%) were aged 6 months or less and 62 (38%) were aged 1 month or less (Table 4). Only 42 *B. pertussis* PCR-positive patients were tested for RSV and of these 2 were positive. None of the *B. parapertussis*-positive patients were tested for RSV.

The total number of samples referred from our laboratory to the NVRL for RSV testing from September 2003 to December 2009 was 5719, and 1108 (19.4%) of these were positive. This indicates a higher incidence of RSV in those patients suspected of having pertussis. Analysis of RSV-positive patients showed that 70% were aged 6 months or less while 54% of the total requests for RSV were from patients 6 months or less. While no seasonal distribution was evident for pertussis, 78% of RSV positives were in the winter months, November to January.

### DISCUSSION

This work describes the development of real-time PCR assays for the routine screening of paediatric respiratory specimens for *B. pertussis* and *B. parapertussis*. The

### Table 4. RSV requests and positives

<table>
<thead>
<tr>
<th>Age</th>
<th>Total RSV requests</th>
<th>Total RSV positive (%)</th>
<th>RSV and pertussis tested patients</th>
<th>RSV-positive pertussis tested patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤1 month</td>
<td>1158</td>
<td>365 (32)</td>
<td>158</td>
<td>62 (39)</td>
</tr>
<tr>
<td>2–6 months</td>
<td>1926</td>
<td>411 (21)</td>
<td>224</td>
<td>67 (30)</td>
</tr>
<tr>
<td>7 months–11 years</td>
<td>2501</td>
<td>328 (13)</td>
<td>165</td>
<td>36 (22)</td>
</tr>
<tr>
<td>12–15 years</td>
<td>100</td>
<td>4 (4)</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>&gt;15 years</td>
<td>34</td>
<td>0 (0)</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>5719</strong></td>
<td><strong>1108 (19)</strong></td>
<td><strong>555</strong></td>
<td><strong>165 (30)</strong></td>
</tr>
</tbody>
</table>
resulting assays are highly sensitive, capable of detecting just one copy of the gene targets per reaction. Consistent with the ‘consensus recommendations’ for use of PCR in the diagnosis of pertussis (Meade & Bollen, 1994), two assays were designed for the detection of B. pertussis, with clinical samples only reported as positive for B. pertussis when duplicate wells for both gene targets were detected. B. holmesii contains the IS481 insertion sequence (Reischl et al., 2001) and as expected, genomic DNA of B. holmesii was detected with the assay targeting B. pertussis IS481, albeit with much lower sensitivity. Mazenga et al. (2000) have reported the isolation of B. holmesii from patients with pertussis-like symptoms; however, B. holmesii was not detected in nasopharyngeal swabs from Finnish and Dutch patients with suspected pertussis (Antila et al., 2006).

The PCR assay targeting the pertussis toxin promoter was designed to fulfil the ‘consensus recommendations’ for use of PCR in the diagnosis of pertussis (Meade & Bollen, 1994), facilitating the differentiation of B. pertussis and B. holmesii in samples positive for the IS481 target. PCR assays targeting the B. pertussis toxin promoter have been reported to be consistently less sensitive than PCR assays for the multi-copy pertussis IS481 target (Riffelmann et al., 2005). Consistent with these findings, serial dilutions of genomic DNA tested in this study, using both PCR assays, identified a 3–8 cycle difference across the dilution series, with the PCR assay targeting the IS481 becoming positive first. Significant levels of sequence homology are observed between the B. pertussis and B. bronchiseptica toxin promoter gene sequences. Primers and probes targeting the B. pertussis toxin promoter were designed maximizing the difference between the B. bronchiseptica and B. pertussis primer and probe annealing regions. The resulting assay detects B. bronchiseptica, albeit with much lower sensitivity.

Thus clinical samples containing B. bronchiseptica or B. holmesii may yield positive results with the B. pertussis toxin promoter or B. pertussis IS481 respectively. However, this does not in any way compromise the detection of B. pertussis, which requires the detection of both gene targets for the reporting of a positive result.

The real-time PCR assays developed in this study resulted in a 94% increase in the detection rate in samples containing B. pertussis when compared to culture and a 91% increase in the detection of positive patients. It is noteworthy however that per-nasal swabs were first cultured on charcoal chocolate and blood agar and therefore quite a lot of original material would have been removed from the swab before the PBS was inoculated. Of the 29 PCR-positive nasopharyngeal samples only 10 were culture positive. The real-time PCR thus resulted in a 290% increase in the detection rate in these samples, where equivalent amounts of material were processed for PCR and culture. Kösters et al. (2001) reported that 8% of symptomatic patients were culture positive while 30.4% were PCR positive. Chan et al. (2002) reported a PCR detection rate of 17% and culture detection rate of only 1.7%. In this study, 5.7% of patients were culture positive while the detection rate by PCR was 10.95%. Fry et al. (2009) reported that 18.9% of samples tested using the same targets but different assays were positive by PCR. In their study samples were limited to infants ≤6 months of age that had been admitted to a paediatric intensive care unit, while in our study 41% were >6 months old and all samples, regardless of source, were included.

The factors affecting culture results include age, stage of illness, specimen quality and transport, culture conditions, prior pertussis vaccination, concurrent antibiotic therapy, and time elapsed since cough onset (Mattoo & Cherry, 2005; Lievano et al., 2002). Culture has previously been reported to be most sensitive in young unvaccinated infants with short duration of symptoms and lowest for adolescents and adults (Wirsing von König et al., 2002). In our study 81% of patients that were positive for PCR were infants ≤6 months of age, and 34% were <1 month old. The incidence of B. parapertussis in this patient group was found to be low, at <0.8%.

The majority of PCR-positive samples were from patients aged <6 months, and patients ≤1 month old accounted for 34% (49) of the total PCR-positive patients (Table 3). It has been reported that the incidence of pertussis in adults and older children is increasing: Celentano et al. (2005) reported a 115% increase in infection in individuals >14 years of age in a 5-year study of cases in Western Europe from 1998 to 2002.

The epidemiology of pertussis has recently been studied and reviewed (Cherry, 2005; Mattoo & Cherry, 2005). Vaccine-induced immunity after three doses lasts for at least 5 years, after which there is a gradual decrease in immunity (Riffelmann et al., 2008). Mooi et al. (2009) reported B. pertussis strains with increased toxin production associated with pertussis resurgence. B. pertussis infection is the cause of 13–20% of prolonged cough in adults and adolescents (Cherry, 2005) and often goes unrecognized, providing a reservoir of infection for unvaccinated or incompletely vaccinated infants. Most cases in this study were unvaccinated or incompletely vaccinated infants. In Ireland, in accordance with the current national immunization schedule, pertussis vaccination is given as part of the 5-in-1 Diphtheria, Tetanus, acellular Pertussis/Haemophilus influenzae type b/Inactivated Polio vaccine (DTaP). The primary course is given at 2, 4 and 6 months, with a booster dose at 4–5 years (Shahadapuri et al., 2004).

In a carefully controlled study, an acellular vaccine was shown to be protective and safe for use in adults and adolescents (Ward et al., 2005) and the Global Pertussis Initiative Group has recommended the use of booster immunization in adolescents (Plotkin, 2005). In Ireland, a booster dose for adolescents has been recommended since 2008 by the National Immunization Programme, but this has not been implemented. Most adults in Ireland would therefore have little immunity to pertussis. In France and
Pertussis infection may be confused with viral respiratory infection. The initial presentation in infants with pertussis is frequently apnoea (Mattoo & Cherry, 2005). A fulminant course of pertussis may develop in very young incompletely immunized infants. Fulminant pertussis is characterized by the development of pneumonia that rapidly evolves to respiratory failure requiring cardiovascular support (Sawal et al., 2009). Life-threatening apnoea may occur in very young infants with viral respiratory infection.

RSV is the most common cause of viral lower respiratory tract infection in infants and young children (van Woensel et al., 2003). Our results suggest that patients who were suspected of having *B. pertussis* were more likely to have RSV infection. Occurrence of dual infection with RSV and *B. pertussis* was reported previously (Korppi & Hiltunen, 2007; Cosnes-Lambe et al., 2008; Walsh et al., 2008). Similarly dual infection occurred in two patients in our study; however it is noteworthy that not all pertussis-positive patients were investigated for respiratory viruses. The difficulty of distinguishing between the two infections is confounded by the fact that the age distributions of RSV infection and *B. pertussis* infection are highly similar. RSV infection is observed to be seasonal while *B. pertussis* infection is not.

In conclusion, this work reports the design, development and application of real-time PCR assays for the rapid, specific and highly sensitive detection of *B. pertussis* and *B. parapertussis* in paediatric clinical specimens. The evidence would suggest that infants and young children suspected of having pertussis should also be screened for RSV. The application of these PCR assays to the routine laboratory repertoire will provide accurate information about the true incidence and epidemiology of pertussis infection in Ireland.

**ACKNOWLEDGEMENTS**

The authors wish to thank the staff of the microbiology laboratory at Our Lady’s Children’s Hospital for technical support and Dr. Norman Fry for helpful advice on the completion of the manuscript.

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