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 $\geq 90\%$. 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.

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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 (Shahdarpuri *et al.*, 2004). The purpose of this study was

Abbreviations: RSV, respiratory syncytial virus.

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, M22031, L26973, S66937 and S66929. GenBank accession numbers AF157332–AF157344 were used for *B. pertussis* toxin promoter real-time PCR assay design. The GenBank accession numbers of *B. bronchiseptica* toxin promoter sequences 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 QIAamp DNA mini kit:

Bordetella petrii NCTC 13317, *B. bronchiseptica* NCTC 10539, *B. avium* NCTC 12033, *B. ansorpii* NCTC 13364, *B. pertussis* NCTC 10739, *B. pertussis* NCTC 13251, *B. trematum* 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. cassiliflavus* 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 (CGTATTACCGCGGCTG, 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 (10⁶–10⁰ 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 C_t (threshold cycle) and the highest ΔR_n (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

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

Name	Sequence (5'–3')*	Position†	Concn (µM)‡
Pertussis IS481 Fwd	CAAGGCCGAACGCTTCAT	862–879	0.05
Pertussis IS481 Rev	AGTTCTGGTAGGTGTGAGCGTAAG	903–926	0.05
Pertussis IS481 probe	6-FAM-CAGTCGGCCTTGCGT-MGB	881–895	0.05
Pertussis toxin prom Fwd	TTCGTCGTACAAAACCCCTCGA	60–81	0.60
Pertussis toxin prom Rev	GTTTCATGCCGTGTTGGATTG	104–123	0.60
Pertussis toxin prom probe	6-FAM-CTTCCGTACATCCC-MGB	83–96	0.20
Parap IS1001 Fwd	CCGGCTCGACGAATTGC	952–968	0.30
Parap IS1001 Rev	AGAACCAGAGCCGTTTGAGTT	1013–1034	0.30
Parap IS1001 probe	6-FAM-AGCCAACCAGCCGCT-MGB	973–986	0.20

*6-FAM, 6-carboxyfluorescein; MGB, minor groove binder.

†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; and accession number X66858 for parapertussis IS1001.

‡Oligonucleotide concentration used real-time PCR amplification reactions.

(Lievano *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-*N*-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 clinical specimens, and extracted samples with a β -actin C_t >36 cycles were reported as unsuitable for *B. pertussis* and *B. parapertussis* PCR. Standard curves prepared with the plasmid DNA controls served as a measure of PCR amplification efficiency and checked that no deterioration had occurred in the primers or probes. Known positive samples were used as positive extraction controls.

Patient samples. All per-nasal swabs and nasopharyngeal aspirates received for pertussis culture between September 2003 and December 2009 were included in the study. Per-nasal swabs that were taken in house were rayon-tipped flexible wire swabs in Amies with charcoal transport medium (Copan).

Bacterial culture of patient samples. Nasopharyngeal aspirates and per-nasal swabs were cultured on charcoal blood agar containing cephalixin (40 µg ml⁻¹), chocolate agar and blood agar within 1 h of

receipt in the laboratory. The samples were then inoculated into 1 ml PBS and frozen at –20 °C for molecular analysis by real-time PCR. Charcoal agar plates were incubated in a moist atmosphere in air for 5 days at 37 °C. Plates were checked at 3 and 5 days. From November 2007 plates were incubated for 7 days with no additional yield. Suspect colonies were identified by standard procedures (Parton, 2005).

Extraction of DNA from patient samples. DNA was extracted from 0.5 ml of the inoculated PBS using the QIAamp DNA mini kit according to the manufacturer's instructions, or the Qiagen M48 biorobot, the MagAttract DNA Mini M48 kit, and the infectious diseases protocol (QIAsoft M Operating System software Version 2.0; Qiagen). Specimen elution was performed in 100 µl of nuclease-free water.

Throat swabs from 20 healthy volunteers were collected, inoculated into PBS and processed in the same way as the clinical samples.

RSV infection. To determine if patients had samples referred for direct immunofluorescence detection of RSV the records of patients who were investigated for pertussis were reviewed on the Laboratory Information System. Only virology requests taken within 1 week of the sample for pertussis culture were included in the analysis. The number tested and the number positive for RSV were determined for each of the following age groups: ≤1 month, 2–6 months, 7 months–11 years, 12–15 years and >15 years. The total number of patients

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

Name	Sequence (5'–3')	Position*	Amplicon length (bp)
Pertussis IS481 Fwd ext	TCAAGGACGCAGTGGCCTACTA	702–723	342
Pertussis IS481 Rev ext	GGTTGTATTTCGTCCAGGTTGAG	1022–1043	
Pertussis toxin prom Fwd ext	GAAACCGCGCCCAAGCT	480–496	218
Pertussis toxin prom Rev ext	CGGACGGTGACCGGTACCAT	148–167	
Parapertussis IS1001 Fwd ext	CGCCCAACGCATCAA	697–711	445
Parapertussis IS1001 Rev ext	CCAGTGGTTCCAGGCTTGTCT	1055–1075	

*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 = 39.40; *B. pertussis* toxin promoter, slope = -3.57 and y -intercept = 39.66; *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 = $(10^{-1/\text{slope}} - 1) \times 100$] and ranged from 90 % to 91 %. A detection limit of ≤ 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. ansorpii* 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. ansorpii* 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 assay 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 ΔR_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. ansorpii* 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: β -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*

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

Age	Total patients	<i>B. pertussis</i>		<i>B. parapertussis</i>	
		PCR positive (%)	Culture positive (%)	PCR positive (%)	Culture positive (%)
≤1 month	338	49 (14.5)	29 (8.5)	0	0
2–6 months	441	68 (15.4)	33 (7.5)	2	2
7 months–11 years	503	27 (5.4)	13 (2.6)	8	5
12–15 years	24	1	1	0	0
>15 years	18	0	0	0	0
Total	1324	145 (10.95)	76 (5.7)	10 (0.8)	7 (0.5)

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 β -actin was > 36 . These samples also tested negative for the presence of *B. pertussis* and *B. parapertussis*.

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 those 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

Age	Total RSV requests	Total RSV positive (%)	RSV and pertussis tested patients	RSV-positive pertussis tested patients (%)
≤1 month	1158	365 (32)	158	62 (39)
2–6 months	1926	411 (21)	224	67 (30)
7 months–11 years	2501	328 (13)	165	36 (22)
12–15 years	100	4 (4)	4	0
>15 years	34	0 (0)	4	0
Total	5719	1108 (19)	555	165 (30)

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. Mazengia *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 (Shahdadi *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

Germany DTaP is now given to adolescents between ages 11–13 and 14–16 respectively. In Australia a fourth dose of vaccine is given in mid-adolescence, instead of at 18 months of age (Halperin, 2005).

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.

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