Role of PCR in the diagnosis of pertussis infection in infants: 5 years' experience of provision of a same-day real-time PCR service in England and Wales from 2002 to 2007

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As part of an enhanced surveillance programme for pertussis in England and Wales, a real-time PCR service for the detection of *Bordetella pertussis* was introduced for infants aged ≤ 6 months admitted to a paediatric intensive care unit or paediatric ward with a respiratory illness compatible with pertussis. Two real-time fluorescent resonance energy transfer hybridization probe LightCycler (Roche Diagnostics) PCR assays were used. One (designed in-house) targeted the pertussis toxin S1 promoter (*ptxA-pr*), and included an internal process control to test for sample inhibition and reagent performance. The other (already published) targeted the insertion element IS481. The analytical sensitivities of the assays were 100 and 10 fg per reaction for the *ptxA-pr* and IS481 PCRs, respectively. The *ptxA-pr* assay was specific for *B. pertussis*, whilst the IS481 PCR also showed some cross-reactivity with *Bordetella holmesii* and the type strain of *Bordetella parapertussis*. From April 2002 to March 2007, 848 samples were received from 774 patients and DNA was extracted. Of 824 samples that were suitable for testing, 183 (22.2%) had evidence of *Bordetella* infection (18.9% *ptxA-pr* and IS481; 3.3% IS481 only), 621 (75.4%) were negative and 20 (2.4%) were inhibitory for the PCR. Within the targeted age group of ≤ 6 months, most patients (130/138) with evidence of *Bordetella* spp. by PCR were ≤ 3 months old. The overall percentage increase in laboratory-confirmed cases due to PCR compared with culture for the 5 year period described ranged from 9 to 26% per year (mean 19%). Real-time PCR is an invaluable tool both for enhanced epidemiological surveillance and for the provision of a rapid diagnosis of pertussis where results can affect patient and contact management.

Received 27 January 2009

Accepted 9 April 2009

INTRODUCTION

Infection with *Bordetella pertussis* (pertussis or whooping cough) causes significant morbidity and mortality and is one of the least-well-controlled vaccine-preventable diseases (Ward *et al.*, 2005). Pertussis remains an important cause of infant death worldwide and is still of concern, even in countries with high vaccination coverage (WHO, 2005). Mild pertussis may be unrecognized in adults, and such adults may act as a source of infection for those most at risk (infants <1 year) (Nelson, 1978; Herwaldt, 1991;

Crowcroft *et al.*, 2003; Senanayake, 2007). In England and Wales, pertussis is a notifiable disease, and the level of notifications fell from 883 in 2002 to a historic low of 409 in 2003. Since then, numbers have increased to between 500 and 600 per year in 2004–2006, but in 2007 this number rose to 1089 (HPA, 2008). However, the main increase in notifications has been in the 15 years and older age group, and is probably due to improved ascertainment due to greater awareness and a consequent increase in laboratory testing (Harnden *et al.*, 2006; Litt *et al.*, 2006; HPA, 2007). Laboratory confirmation of pertussis infection can be made by isolation of the causative organism, *B. pertussis*; demonstration of the presence of genomic DNA from the organism in clinical samples, typically nasopharyngeal aspirates (NPAs) and pernasal swabs (PNSs), by

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Abbreviations: IPC, internal process control; NPA, nasopharyngeal aspirate; PNS, pernasal swab; WHO, World Health Organization.

PCR; and estimation of the levels of specific antibodies to pertussis by serological or oral fluid assays (Giammanco *et al.*, 2003a, b; Litt *et al.*, 2006; André *et al.*, 2008). Whilst culture of *B. pertussis* is to be encouraged and is traditionally viewed as the definitive diagnosis of pertussis, it can be slow and of varying, often low, sensitivity (Dragsted *et al.*, 2004; Bamberger *et al.*, 2005). In the absence of a vaccine history, serological tests – or more recently oral fluid antibody tests – cannot usually distinguish between recent vaccination against pertussis and recent infection, although they are still useful in providing a retrospective diagnosis, particularly for older children and adults, and can be more sensitive than culture (Kerr & Matthews, 2000; Litt *et al.*, 2006).

The utility of PCR for the diagnosis of pertussis is now well established and has been shown to have improved sensitivity over culture (Grimprel *et al.*, 1993; He *et al.*, 1993; Muyldermans *et al.*, 2005). In addition, improvements in nucleic acid extraction and real-time PCR technologies have resulted in shorter assay turnaround times (Reischl *et al.*, 2001a; Muyldermans *et al.*, 2005). Various genomic targets have been described, including those targeting the promoter region of the gene encoding the pertussis toxin S1 subunit (*ptxA*) (Houard *et al.*, 1989; Mastrantonio *et al.*, 1996), the insertion element IS481 (Glare *et al.*, 1990), the adenylate cyclase-encoding gene (*cyaA*) (Douglas *et al.*, 1993) and a region upstream of the outer-membrane porin gene (Li *et al.*, 1994). Several of these assays have been implemented on real-time platforms (Muyldermans *et al.*, 2005).

An enhanced surveillance scheme for pertussis infection (based on laboratory-confirmed cases with epidemiological follow-up) was established in 1994 for England and Wales by the former Public Health Laboratory Service (Van Buynder *et al.*, 1999) and is now provided by the Health Protection Agency. The purpose of this scheme is to monitor pertussis infection and vaccine efficacy in order to enable informed decisions to be made with respect to vaccine policy (e.g. to assess the need for booster vaccinations). As part of this scheme, in addition to capturing data from culture-confirmed cases, new diagnostic methods (serodiagnosis and PCR detection) were introduced by the Respiratory and Systemic Infection Laboratory and officially launched in April 2002.

This study reports on the experience of a reference laboratory using a real-time PCR assay for *B. pertussis* adapted from the previously described block-based thermocycler PCR assay using detection of products by gel electrophoresis (Fry *et al.*, 2004). This real-time PCR service was applied to clinical specimens as a same-day service for pertussis diagnosis in infants (≤ 6 months) over a 5 year period from April 2002 to March 2007. Where other samples were submitted from these patients (i.e. *B. pertussis* isolates for confirmation), these data are also presented.

METHODS

Patients, specimens and inclusion criteria. The data presented are from specimens received between 1 April 2002 and 31 March 2007. Inclusion criteria for the offered *B. pertussis* PCR service were: NPAs or PNSs (collected with a fine, flexible, twisted Nichrome wire shaft swab and rayon bud) not previously used for culture, from infants ≤ 6 months of age with respiratory illness compatible with pertussis infection, admitted to paediatric intensive care units or paediatric wards. Samples fulfilling these criteria received by 10 a.m. (Monday to Friday) were processed and reported the same day. Specimens and resulting data from outside these criteria (not routinely tested on the same day, but in the next available run) are noted as such in the text. Where available, relevant data from additional samples from these patients (i.e. putative *B. pertussis* isolates for confirmation and serotyping) are also presented. Clinical samples submitted to the PCR service were extracted as described below and our laboratory did not attempt to isolate *B. pertussis* directly from these samples. The identity of putative *B. pertussis* isolates submitted for confirmation and serotyping was established using standard methods (Fry *et al.*, 2004).

DNA extraction. The reference strains *B. pertussis*, *Bordetella avium*, *Bordetella bronchiseptica*, *Bordetella hinzii*, *Bordetella holmesii*, *Bordetella parapertussis*, *Bordetella trematum*, *Bordetella petrii* and '*Bordetella ansorpii*' were obtained from the National Collection of Type Cultures (UK) and the Culture Collection, University of Göteborg (Sweden). Genomic DNA used for positive controls or specificity experiments from members of the genus *Bordetella* (Von Wintzingerode *et al.*, 2001) was prepared as described previously (Fry *et al.*, 2004). Previously extracted genomic DNA from other genera and species (*Bartonella birtlesii*, *Bartonella bacilliformis*, *Chlamydia* spp., *Chlamydiophyla* spp., *Citrobacter* spp., *Corynebacterium diphtheriae*, *Coxiella* spp., *Enterococcus faecalis*, *Escherichia coli*, *Haemophilus influenzae*, *Klebsiella* spp., *Legionella pneumophila*, *Legionella bozemaniae*, *Neisseria meningitidis*, *Mycoplasma orale*, *Mycoplasma pneumoniae*, *Proteus* spp., *Pseudomonas aeruginosa*, *Ureaplasma* spp., *Staphylococcus aureus*, *Streptococcus anginosus*, *Streptococcus pyogenes*, *Streptococcus intermedius*, Lancefield group G *Streptococcus*, Lancefield group B *Streptococcus*, *Streptococcus salivarius*, *Streptococcus bovis*, *Streptococcus gordonii*, *Streptococcus oralis* and *Gemella haemolysins*) were obtained from the authors' laboratory (Respiratory and Systemic Infection Laboratory). Genomic DNA suitable as template for the *B. pertussis* PCR assays was extracted from clinical specimens, including respiratory samples (usually NPAs and PNSs) or tissue samples using a QiaAmp DNA mini kit (Qiagen) or MagNaPure Compact (Roche) according to the instructions of the manufacturer. To control for possible contamination of the extraction procedure, a negative control comprising 200 μ l PBS was subjected to the same extraction procedure with each batch of clinical samples. Typically, a volume of 200–400 μ l NPA was extracted. Where the starting volume was less than 200 μ l, it was made up to 200 μ l with nuclease-free water (Sigma). For PNSs, the flexible wire shaft was cut approximately 1.5 cm from the swab tip with sterile scissors and the swab was vortexed in 200 μ l PBS, expressed on the side of the tube and removed using sterile forceps prior to extraction of the solution. DNA extracts were eluted in 100 μ l nuclease-free water and were either assayed immediately or stored at 4 °C. Long-term storage of extracts was at –25 °C or below. Extracted clinical samples were tested at neat, 1 : 10 and 1 : 100 dilutions in nuclease-free water (Sigma).

Construction of an internal process control (IPC) for detection of inhibition. The presence or absence of PCR inhibitors and the performance of the PCR assay in extracted specimens were determined using an IPC. The IPC was constructed to contain bacteriophage λ DNA within sequences homologous to the two

primers used in the *ptxA*-pr assay. Initially, a PCR product was amplified using λ DNA as a template, and two primers specifically designed to incorporate both λ DNA and the *ptxA*-pr assay primer sequences (Table 1). This product was then cloned into a TOPO plasmid vector (TOPO TA cloning kit; Invitrogen) and the construct was transformed into competent *Escherichia coli* One Shot TOP10 cells (Invitrogen). Transformants were subcultured on Luria–Bertani medium containing 50 μ g ampicillin ml⁻¹ (Sigma), and purified plasmid DNA was prepared using a QIAprep miniprep kit (Qiagen). Plasmid preparations were linearized using a restriction endonuclease with a single site in the vector and none within the cloned region (e.g. *Xba*I), and re-precipitated with 3 M sodium acetate (pH 5.2) at a final concentration of 0.3 M and ethanol (Sambrook & Russell, 2001). Linearized plasmid IPC was titrated in the *ptxA*-pr assay to achieve a crossing point of approximately 33 cycles (~45 copies, 0.2 fg per reaction mix) with probes IPC-DNR and IPC-ACR (Table 1) targeted against the internal λ DNA. A linear (non-exponential) result in the absence of any positive signal with *ptxA* or IS481 was scored as inhibitory.

B. pertussis LightCycler PCR assays targeting the B. pertussis toxin promoter together with an IPC and targeting the insertion element IS481. A consensus (dual-target) PCR approach was used; LightCycler (Roche Diagnostics) assays for two gene targets were designed using fluorescence resonance energy transfer hybridization probes for confirmation of products. The donor fluorophore, fluorescein, was used to label one of each pair of probes at the 3' terminus, and the acceptor fluorophore, LC-Red 640 or LC-Red 705, was used to label the other at the 5' terminus (Table 1). The first assay was designed in house and targeted the pertussis toxin S1 (*ptxA*) promoter region (*ptxA*-pr PCR). This assay comprised one pair of primers targeting the *B. pertussis* *ptxA* promoter and two pairs of hybridization probes, one pair designed to target a region internal to the *ptxA*-pr product, and one pair specific for the λ sequence within the IPC as described above (Table 1). The second assay (IS481 PCR), described by Reischl *et al.* (2001a), comprised one pair of primers targeting the insertion sequence (IS481) of *B. pertussis* and one pair of hybridization probes. This PCR targeted the insertion element IS481 (which occurs in *B. pertussis*, *B. holmesii* and some strains of *B.*

bronchiseptica and *B. parapertussis*) (Reischl *et al.*, 2001a, b; Kösters *et al.*, 2002). All reaction mixtures were in a total volume of 20 μ l and comprised 1 $\times$ LightCycler FastStart DNA master hybridization probes mix (Roche Diagnostics), 0.5 μ M each primer, 0.25 μ M each probe and 1 U heat-labile uracil-DNA glycosylase (Roche). The two assays included final concentrations of 4.0 mM MgCl₂ (Roche) for the *ptxA*-pr assay and 3.0 mM MgCl₂ for the IS481 assay. The *ptxA*-pr assay also contained approximately 45 copies (0.2 fg) of the *ptxA*-pr IPC. Amplification and detection were performed with a LightCycler (using LightCycler software version 5.32). The cycling conditions used were based on those described by Reischl *et al.* (2001a) with the addition of a melt and cooling step: initial denaturation for 10 min at 95 °C, followed by 50 cycles of denaturation for 10 s at 95 °C, annealing for 10 s at 50 °C and extension for 20 s at 72 °C; a melt step of 95 °C for 0 s, 40 °C for 30 s and 95 °C for 0 s (with a ramping rate of 0.1 °C s⁻¹); and a final cooling step of 40 °C for 30 s. Except where stated otherwise, all ramping rates were maximal (20 °C s⁻¹). Fluorescence was measured using the single acquisition mode during the annealing step or continuous mode in the third segment of the melt. All extracted specimens were tested in the LightCycler PCR assays using 5 μ l undiluted extract, together with 5 μ l of 1:10 and 1:100 dilutions (in nuclease-free water). Positive controls of purified *B. pertussis* DNA were included, using at least four dilutions for each assay (100, 10, 1 and 0.1 pg for the *ptxA*-pr assay, and 10, 1, 0.1 and 0.01 pg for the IS481 LightCycler assay) in order to calculate a standard curve. Additional controls included with each batch of samples tested comprised 5 μ l undiluted extract from a negative control of PBS (extracted as described above) and 5 μ l nuclease-free water (no added DNA).

Interpretation of LightCycler PCR results. Usually, analyses were performed using the automated (default) method with colour compensation for the *ptxA*-pr and IPC results. If required, results were also examined using the fitted-points method. For reporting purposes, the real-time PCR results for *B. pertussis* were divided into five classes: (i) *B. pertussis* positive, defined by a positive amplification signal in both the *ptxA*-pr and IS481 PCRs; (ii) *B. pertussis* negative, defined by the absence of a positive amplification signal in both the *ptxA*-pr and IS481 PCRs, together with a positive signal with the IPC;

Table 1. Oligonucleotides used in this study

Oligonucleotide name	Target	Sequence (5'→3')*	5' label	3' label	Reference
Bp- <i>ptxA</i> -Pr-IPC1-F	<i>ptxA</i> -Pr/ λ	CGCCAAGCTGAAGTAGCAActaacc-tgtacggta	None	None	This study
Bp- <i>ptxA</i> -Pr-IPC1-R	<i>ptxA</i> -Pr/ λ	AAGGAGCGTTTCATGCCGccagcat-gatacgtc	None	None	This study
Bp- <i>ptxA</i> -Pr-225F	<i>ptxA</i> -Pr	CGCCAAGCTGAAGTAGCA	None	None	This study
Bp- <i>ptxA</i> -Pr-397R	<i>ptxA</i> -Pr	AAGGAGCGTTTCATGCCG	None	None	This study
Bp- <i>ptxA</i> -Pr-315F-HP1	<i>ptxA</i> -Pr	CATGCGTGCAGATTCGTCTGATCA	None	FITC	This study
Bp- <i>ptxA</i> -Pr-340F-HP2	<i>ptxA</i> -Pr	TGCAGCGAACTGAGCGCGGT	LC-Red 705	Phosphate	This study
IPC-DNR	IPC	AGCCGCTGGCGCATTGAGCA	None	FITC	This study; Sheppard <i>et al.</i> (2004)
IPC-ACR	IPC	TGCAGCGAACTGAGCGCGGT	LC-Red 705	Phosphate	This study; Sheppard <i>et al.</i> (2004)
Bp-IS481-12F	IS481	GATTCAATAGTTGTATGCATGGTT	None	None	Glare <i>et al.</i> (1990); Reischl <i>et al.</i> (2001a, b)
Bp-IS481-192R	IS481	TTCAGGCACACAACTTGATGGGCG	None	None	Reischl <i>et al.</i> (2001a, b)
Bp-IS481-66F-HP1	IS481	TCGCCAACCCCCAGTTCACTCA	None	FITC	Reischl <i>et al.</i> (2001a, b)
Bp-IS481-112F-HP2	IS481	AGCCCGGCCGGATGAACACCC	LC-Red 640	Phosphate	Reischl <i>et al.</i> (2001a, b)

FITC, Fluorescein isothiocyanate.

*The pertussis toxin promoter sequence is in upper case and the λ sequence in lower case.

(iii) *Bordetella* spp. positive, defined by a positive amplification signal in the IS481 PCR only; (iv) inhibitory, defined by a non-exponential/linear amplification signal with the IPC; and (v) equivocal, defined by a positive amplification signal in the *ptxA*-pr PCR only. It should be noted that, due to the greater sensitivity of the IS481 assay, a positive signal in this assay without a corresponding signal in the *ptxA*-pr assay could be due to either very low amounts of *B. pertussis* or to the presence of another *Bordetella* species such as *B. holmesii* (Reischl *et al.*, 2001b), *B. bronchiseptica* or *B. parapertussis*. In equivocal cases, further specimens for PCR and/or serological investigation were requested if appropriate.

RESULTS AND DISCUSSION

PCR sensitivity and specificity

The analytical sensitivities of the *Bordetella* PCR LightCycler assays were determined to be 100 and 10 fg per reaction for the *ptxA*-pr and the IS481 PCRs, respectively. Although the sensitivity of the *ptxA*-pr could reach 10 fg, because this level of detection was not consistently achieved, the sensitivity of this assay was taken to be 100 fg for reporting purposes. Of the nine species within the genus *Bordetella*, the *ptxA*-pr assay was specific for *B. pertussis* (Table 2).

The IS481 assay as used here was originally described as specific for *B. pertussis* (Reischl *et al.*, 2001a), using a number of strains belonging to five *Bordetella* species and 80 Gram-positive and Gram-negative organisms from other genera. It has since been shown to give a positive signal with *B. holmesii*, and some strains of *B. parapertussis* and *B. bronchiseptica*. We confirmed the cross-reactivity with *B.*

holmesii and also with the type strain of *B. parapertussis* (although in the latter case this had a crossing point of >40 cycles). We extended the specificity experiments in this study by first (re)-testing purified DNA from strains belonging to all nine described species of *Bordetella* and from other genera. Neither assay gave a positive amplification signal with any member of a non-*Bordetella* genus tested. The type strain of *B. pertussis*, which has been found to be atypical in many of its characteristics compared with other *B. pertussis* strains (Packard *et al.*, 2004), did not give an amplification signal with the IS481 assay, presumably due to variation in the primer or probe sites.

Patients, specimens and PCR results

During the study period, 848 samples from 774 patients were submitted for analysis. Single specimens were obtained from the majority (716/774, 92.5 %) of patients, followed by two specimens per patient (52/774, 6.7 %). Occasionally, multiple samples were received: three specimens from each of four patients, and seven and nine post-mortem samples from two patients. Details of the numbers of patients by age and the results obtained by PCR are shown in Table 3. The majority (584/774, 75.5 %) belonged to the target group of ≤ 6 months. The gender was stated for 721/774 patients (93.4 %) from whom samples were received and the ratio was 1.03:1 for males: females. The majority of samples submitted were respiratory samples comprising NPAs (489 samples, 57.7 %), PNSs (249 samples, 29.4 %), endotracheal secretions (24 samples, 2.8 %), bronchoalveolar lavages (30 samples, 3.5 %), throat and nose swabs (15 samples, 1.8 %) and sputum (5 samples, 0.6 %). Other specimen types submitted included tissue (13 samples, 1.5 %), cerebrospinal fluid (1 sample, 0.01 %), EDTA blood or sera (7 samples, 0.8 %) and some samples whose nature or site was not specified (scored as not known) (15 samples, 1.8 %).

Detection of *Bordetella* by PCR

We used a consensus approach together with an internal control in order to provide greater confidence in the results

Table 2. Specificity of PCR assays for members of the genus *Bordetella*

Species	<i>ptxA</i> -pr	IS481
<i>B. pertussis</i> NCTC 10739 ^T	+	–
<i>B. pertussis</i> NCTC 8189	+	+
<i>B. avium</i> NCTC 12033 ^T	–	–
<i>B. avium</i> NCTC 12034	–	–
<i>B. bronchiseptica</i> NCTC 452 ^T	–	–
<i>B. bronchiseptica</i> NCTC 458	–	–
<i>B. parapertussis</i> NCTC 5952 ^T	–	+*
<i>B. parapertussis</i> NCTC 10853	–	–
<i>B. hinzii</i> NCTC 13199 ^T	–	–
<i>B. hinzii</i> NCTC 13200	–	–
<i>B. holmesii</i> NCTC 12912 ^T	–	+
<i>B. holmesii</i> NCTC 13202	–	+
<i>B. trematum</i> NCTC 12995 ^T	–	–
<i>B. trematum</i> NCTC 13203	–	–
<i>B. petrii</i> CCUG 43448 ^T	–	–
<i>B. petrii</i> NCTC 13363	–	–
' <i>B. ansorpii</i> ' NCTC 13364 ^T	–	–

CCUG, Culture Collection University of Göteborg; NCTC, National Collection of Type Cultures.

*Crossing point value >40.

Table 3. Results of testing 774 patients by *B. pertussis* PCR from April 2002 to March 2007

Age group	No. of patients	No. of patients with evidence of <i>Bordetella</i> spp. by PCR (%)
≤ 6 months	584	138 (23.6)
7–11 months	52	4 (7.7)
1–10 years	82	19 (23.2)
11–20 years	19	3 (15.8)
21–66 years	25	2 (8.0)
Unknown	12	2 (1.7)
Total	774	168 (21.7)

obtained. Two independent targets were used (*ptxA*-pr and IS481) and an IPC was used to test for the presence of PCR inhibitors and to confirm the integrity of the assay. From April 2002 to March 2007, 848 samples were received from 774 patients for investigation by *B. pertussis* PCR. Twenty-four samples (2.8%) were not tested by PCR for various reasons, e.g. unsuitable sample due to age of sample, duplicate or incorrect sample type such as EDTA blood or serum. Of the 824 samples tested, 183 (22.2%) had evidence of *Bordetella* spp. (18.9% *ptxA*-pr and IS481; 3.2% IS481 only), whilst 621 (75.4%) were negative and 20 (2.4%) were inhibitory. The inhibitory samples included 14/489 NPAs, 5/249 PNSs and 1/30 bronchoalveolar lavages, but no statistically significant association of inhibition and sample type was found. Some samples contained PCR inhibitors resulting in either total (scored as inhibitory) or partial inhibition. Typically, dilution of the extracted DNA removed or reduced the inhibitory effect allowing the sample to be scored as negative or positive, but with a concomitant reduction in the sensitivity of the assay. The crossing point values for samples positive for both targets were usually at least 3.3 cycles higher in the IS481 assay corresponding to at least a tenfold increase in sensitivity. In samples positive for the IS481 only, crossing points were usually observed after 35 cycles. In one case where sequential samples were obtained from an infant transferred between hospitals, the earlier specimen taken prior to antibiotic therapy was both *ptxA*-pr and IS481 positive, whilst the later one was IS481 positive only.

In special circumstances, post-mortem samples from tissue samples (including lung, liver, kidney and spleen) were investigated from four patients, one of whom was a case of sudden infant death syndrome (SIDS). One (non-SIDS) patient was PCR positive (*ptxA*-pr and IS481). Blood and sera samples are considered inappropriate samples for *B. pertussis* PCR investigation and these samples were not tested by this method; however, estimation of anti-pertussis toxin IgG antibodies in such samples was undertaken if appropriate (i.e. if the patient had not been vaccinated recently). For PCR, respiratory samples were either processed immediately or held at -80°C before genomic DNA extraction.

Results from patients' samples submitted in addition to those investigated by PCR

Putative *B. pertussis* isolates (for confirmation and/or serotyping) were received from a number of patients tested by PCR. Of 36 *B. pertussis* PCR-positive patients, isolates from 17 of these patients were confirmed as *B. pertussis* by culture. There was 1 *B. pertussis* culture-positive sample from 30 patients that were PCR negative for *B. pertussis*, but the specimen submitted (a throat swab) to our laboratory for PCR from this patient was one not considered suitable for the collection of *B. pertussis*.

The PCR results from this service, together with confirmed *B. pertussis* isolates and serological data, comprise the

laboratory-confirmed cases of pertussis reported to the pertussis enhanced surveillance scheme. Despite the limitations of the PCR service described, i.e. restricted target age and service not offered out of regular working hours, we believe it has provided real benefits in a number of ways, including a more rapid turnaround time. The previous block-based methodology took a mean time of 3 h per PCR run, making the whole assay process from receipt of specimen to reporting of result difficult to fit into a working day. In addition, although the mean number of days from sample collection to receipt was 3.6 days, the modal number of days was 1. For primary isolation, *B. pertussis* can grow on *Bordetella*-selective medium (charcoal blood agar with cephalaxin) within 3 days, but 5–7 days may be required. Moreover, it is recommended that inoculated plates are incubated for 7 days before being discarded as negative (Matthews, 1997). For samples delivered to us within 24 h of collection, a result on the same day as receipt was routinely provided, offering a real opportunity to affect patient management. Inclusion of an IPC prevents the reporting of false-negative results and effectively controls the PCR process. The rationale for using a consensus (two-target) approach was to ensure complete confidence in the results obtained. However, the superior sensitivity of the IS481 assay due to the presence of IS481 in multiple copies on the *B. pertussis* genome has led to its widespread use, usually as a single-target assay. The presence of *Bordetella* species other than *B. pertussis* in respiratory samples submitted for *B. pertussis* PCR cannot be ruled out, but to date the presence and frequency of these other species, which may give cross-reactivity using IS481 as a target, appear to be low. The recovery of *B. holmesii* from NPA specimens of otherwise healthy young persons with a cough has been demonstrated (Yih *et al.*, 1999), but *B. holmesii* DNA was not found in DNA extracts of NPA swabs from Finnish and Dutch patients with suspected pertussis (Antila *et al.*, 2006). The non-specific nature of IS481 as a target for *B. pertussis* sequences has recently led to the description of new targets for specific detection of this species (Probert *et al.*, 2008). Concern has also been raised over false positives and/or contamination in laboratories performing PCR (Probert *et al.*, 2008). During the course of this study, our laboratory participated in an external quality assessment for the molecular detection of *B. pertussis* in European laboratories (Muyldermans *et al.*, 2005). No false-positive results were reported by any of the six laboratories, although misidentification of *B. holmesii* and *B. bronchiseptica* for the reasons described above was observed. This highlights the value of such external quality assessment schemes and the need for all laboratories involved in PCR testing to participate.

The added value of the PCR can be measured in different ways, but all make assumptions. If one assumes that cases of suspected pertussis would be missed (by lack of laboratory confirmation) in the absence of the *B. pertussis* PCR, the percentage increase due to the PCR, compared

with culture (in the ≤ 6 months group) ranged from 9 to 26% (mean 19%) from 2002 to 2007. Although in some cases *B. pertussis* was isolated after the specimen was found to be PCR positive, and it could be argued that availability of PCR testing for *B. pertussis* may lead to an overall decrease in attempts to culture, the rapid nature of the PCR test provides a considerable advantage. The detection of PCR positives in the older age groups has led us to increase the age range to ≤ 1 year (from April 2007), although the majority of cases are still in the ≤ 6 months group.

In conclusion, real-time PCR is an invaluable tool both for enhanced epidemiological surveillance and for the provision of a rapid diagnosis of pertussis where results can affect patient (and contact) management. Together with serological results, and isolation and confirmation of the identity of putative *B. pertussis* isolates, these data are invaluable in enabling informed decisions to be made with respect to the vaccination programme for pertussis. During the course of this study, our laboratory also assisted in a number of potential outbreak/cross-infection investigations in healthcare workers on paediatric intensive care units and children's wards by providing rapid screening of healthcare workers and infants. We demonstrated an increase in the detection rate of pertussis infection using PCR, compared with culture, in a limited target age group. If PCR detection were to be used routinely for all ages, then more accurate estimates of pertussis infection should be possible. In the future, we anticipate that, increasingly, regional and hospital laboratories equipped for PCR will be able to test for *B. pertussis* and *B. parapertussis* using in-house published or commercial assays. Reference laboratories could provide confirmation of these results, using a species-specific target (as illustrated for *B. pertussis* in this study) and additional typing data, such as multi-locus variable-number tandem repeat analysis directly from PCR-positive material (Alexander *et al.*, 2008; Litt *et al.*, 2009).

ACKNOWLEDGEMENTS

We thank the hospitals and microbiology laboratories for submission of specimens, the staff of the Atypical Pneumonia Unit (Health Protection Agency Centre for Infections) for their technical support and Udo Reischl for helpful advice concerning the IS481 assay.

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