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Laboratory diagnosis of pertussis infections: the role of PCR and serology

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This study reports on practical laboratory aspects of pertussis diagnosis. PCR assays were applied to respiratory specimens obtained during a large study of infants (less than 5 months old) admitted to paediatric intensive care units ($n = 122$), children (less than 15 years old) admitted to paediatric wards ($n = 16$) and their household contacts ($n = 320$). Estimation of antibodies to pertussis toxin and culture for *Bordetella pertussis* were attempted on specimens from the same patients, where available, and the overall utility of the diagnostic PCR assays was assessed by comparison to these results. A PCR assay for the human mitochondrial cytochrome oxidase (HMCO) gene was used for quality control of the extracted samples and an internal process control (IPC) was included in each sample to test for PCR inhibition. Four of 458 samples were considered unsuitable (three HMCO negative, one IPC negative) and excluded from further analyses. Positive PCR results were considered valid if they were either (i) positive for both of two *B. pertussis* gene targets (pertussis toxin S1 promoter and the insertion element IS*481*), i.e. consensus PCR positive, or (ii) repeatably positive in only one assay. Using these criteria, 52 of 454 (11·5 %) samples were considered as PCR positive for *B. pertussis*. Six of 356 samples were culture-positive for *B. pertussis*, 1/88 infants, 3/14 children and 2/254 contacts, giving an overall isolation rate of 1·7 %. Using these data, PCR gave an almost fivefold increase in diagnostic yield compared with culture (McNemar's test; $P < 0{\cdot}0001$). Sera from 9/111 infants, 5/10 children and 14/210 contacts were positive. Serology and PCR results showed a high level of agreement (113/121) for infants and children. PCR demonstrated a significant improvement in diagnostic yield over culture. Serological testing also resulted in a significant increase in diagnostic yield compared to culture alone. PCR is a useful technique, but validity of results must be assured by careful control. Rapid diagnosis of *B. pertussis* infection particularly in infants by PCR, together with serological assays, can enhance surveillance systems for pertussis in all age groups.

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INTRODUCTION

Definitive diagnosis of pertussis has traditionally been made by culture of the causative organism, *Bordetella pertussis*. However, as this approach may be insensitive and slow, taking up to 7 days to obtain a clear result, it is not always attempted and, consequently, pertussis is under-diagnosed (Anonymous, 2003). Serological tests to assist the diagnosis of pertussis are performed but, although more sensitive than culture, these usually only provide late, or retrospective diagnosis (Kerr & Matthews, 2000). To overcome these limitations, detection of *B. pertussis* DNA from naso-pharyngeal aspirates (NPAs) and swabs (NPSs) has been described using PCR assays, including those targeting the promoter region of the gene encoding pertussis toxin S1 subunit (*ptxA*) (Houard *et al.*, 1989; Mastrantonio *et al.*, 1996), the insertion element IS*481* (Glare *et al.*, 1990), the adenylate cyclase gene (*cyaA*) (Douglas *et al.*, 1993) and a

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Abbreviations: IPC, internal process control; HMCO, human mitochondrial cytochrome oxidase; PICU, paediatric intensive care unit.

region upstream of the outer-membrane porin gene (Li *et al.*, 1994). However, few data are available to assess the relative utility of the various diagnostic approaches.

This study reports on the practical aspects of the laboratory diagnosis of pertussis, using in addition to culture, PCR and serology, as applied to clinical specimens obtained as part of a large prospective study of pertussis. Specimens were received from infants admitted to paediatric intensive care units (PICUs), children admitted to paediatric wards and their household contacts. The clinical and epidemiological aspects of this study have been described in detail previously (Crowcroft *et al.*, 2003).

METHODS

Specimens and inclusion criteria. Specimens were collected between November 1998 and October 2000, from eligible infants under 5 months of age admitted to London PICUs, eligible children under 15 years old admitted to paediatric wards and their respective household contacts (Crowcroft *et al.*, 2003). Eligible subjects all had an illness consistent with a diagnosis of pertussis. For the study presented here only those eligible subjects and contacts from whom respiratory samples were available were included in the analysis. All respiratory samples (one per subject) including NPAs, NPSs and endotracheal secretions were examined by PCR, and those samples submitted between 1 November 1998 and 31 October 1999 were also examined by culture (see Table 1). For PCR, respiratory samples were divided into aliquots on arrival. One aliquot was either processed immediately or held at −80 °C for up to 1 week before genomic DNA extraction. A second aliquot (archive sample) was stored at −80 °C for re-examination if the first PCR assay yielded ambiguous results. Samples examined by culture were processed within 4 h of collection. Acute and/or convalescent sera were collected from patients and wherever possible a single serum was collected from adult contacts. Sera were stored at 4 °C until tested.

Bacterial culture conditions. Isolation of *B. pertussis* was attempted by culture of respiratory samples on blood charcoal agar plates containing 40 mg cephalexin μl^{-1} (Media Services, CPHL). Primary isolation plates were incubated at 35–37 °C, in a moist atmosphere, and maintained for 7 days. Plates were examined at 4 and 7 days. Putative colonies consistent with the appearance of *B. pertussis* were tested by Gram's stain, oxidase, catalase, slide agglutination with polyvalent antisera (Difco) and immunofluorescent microscopy using *B. pertussis*-specific mAb (Difco).

DNA extraction. Genomic DNA used for positive controls or specificity experiments from *Bordetella* species (including *B. pertussis*, *Bordetella avium*, *Bordetella bronchiseptica*, *Bordetella parapertussis*, *Bordetella holmesii* and *Bordetella trematum*) were prepared using the Nucleon BACC2 DNA extraction kit (Amersham Life Sciences) including an RNase A treatment. Previously extracted genomic DNA from other genera including *Bartonella* species, *Escherichia coli* and *Mycoplasma* species, were obtained from the authors' laboratory. Genomic DNA, suitable as template in the PCR, was extracted from respiratory specimens using the QIAamp DNA mini kit (Qiagen), according to the manufacturer's instructions. To control for possible contamination of the extraction procedure, a simulated specimen comprising 200 µl nuclease-free water (Promega) was subjected to the same extraction procedure with each batch of clinical samples. Typically a volume of 200–400 µl of respiratory sample was extracted. Where the starting volume was less than 200 µl, it was made up to 200 µl with nuclease-free water (Promega). Genomic DNA extracts were eluted in 200 µl elution buffer (Qiagen) and were either assayed immediately or stored at −80 °C.

Quality control of specimens. To assess the quality of the DNA extracts from clinical specimens, the presence or absence of human DNA was determined using a PCR assay targeting the human mitochondrial cytochrome oxidase (HMCO) gene. Primers (H6A and H6B; Table 2) were used to amplify an 823-bp product of the HMCO gene (Cadieux *et al.*, 1993). Reaction mixtures were in a total volume of 50 µl and contained 2·5 mM $MgCl_2$, 15 mM Tris/HCl (pH 8·3), 50 mM KCl, 200 µM each deoxynucleotide (Roche Diagnostics), 20 pmol each primer (MWG Biotech) and 1·25 U AmpliTaq Gold (PE Applied Biosystems). Thermal cycling conditions were adapted from those described by Cadieux *et al.* (1993); pre-denaturation for 10 min at 95 °C, followed by 30 cycles of denaturation for 1 min at 94 °C, annealing for 1 min at 65 °C and elongation for 1 min at 72 °C, with a final elongation of 5 min at 72 °C.

Samples yielding a band of the expected size (823 bp) in this assay were scored as positive, and were considered as suitable for further testing by the *B. pertussis*-specific PCR assays. Where clinical samples yielded negative results (no band of expected size) in this assay, a second aliquot of the specimen (archive sample) was extracted. If this second specimen also yielded a negative result in this assay, the specimen was scored as unsuitable for further testing. The HMCO assay is known to be an extremely robust PCR (Cadieux *et al.*, 1993; D. Pitcher, personal communication) and thus amplicons are normally still visible even in the presence of PCR inhibitors, due to the large amount of human DNA in clinical samples compared to microbial DNA. Extracts of clinical specimens were tested in the HMCO PCR at 1 : 10 and 1 : 100 dilutions (in nuclease-free water).

Table 1. Age group and number of persons examined

Only patients for whom respiratory specimens were available were included. All of these specimens were examined by *B. pertussis*-specific PCR assays. Culture for *B. pertussis* was attempted and sera were examined for pertussis toxin IgG from this group where indicated.

Group	No. persons for PCR	No. persons for serology	No. persons for culture
Infants (< 5 months) PICU	122	111	88
Children (< 15 years) hospital ward	16	10	14
Household contacts	320	210	254
Total	458	331	356

Table 2. Oligonucleotides used in this study

Primer	Sequence (5′–3′)	Target	Reference
PTp-IPC-F	CCAACGCGCATGCGTGCAGATTCGTCctgacggtttctaac*	λ	J. Jensen (personal communication)
PTp-IPC-R	CCCTCTGCGTTTTGATGGTGCCTATTTTagacatacggaaatag*	λ	J. Jensen (personal communication)
PTp1	CCAACGCGCATGCGTGCAGATTCGTC	*ptxA*-Pr	Houard *et al.* (1989)
PTp2	CCCTCTGCGTTTTGATGGTGCCTATTTTA	*ptxA*-Pr	Houard *et al.* (1989)
BP-1	GATTCAATAGGTTGTATGCATGGTT	IS*481*	Glare *et al.* (1990)
BP-2MOD	TGGACCATTTCGAGTCGACG	IS*481*	J. Jensen (personal communication), modified from Glare *et al.* (1990)
H6A	ATGACCCACCAATCACATGCCTATCA	HMCO	Cadieux *et al.* (1993)
H6B	ACTAGTTAATTGGAAGTTAACGGTACTA	HMCO	Cadieux *et al.* (1993)

*λ sequences are in lower case and the sequences of primers PTp1 and PTp2 are in upper case.

Construction of an internal process control (IPC) for detection of inhibition. Presence or absence of PCR inhibitors in extracted specimens was determined with an IPC using the bacteriophage λ genome. The IPC was constructed to contain λ DNA within sequences homologous to the two primers used in the PCR assay for the pertussis toxin S1 (*ptxA*) promoter (PTp1 and PTp2; Table 2). Initially a PCR product was amplified using λ DNA as template and primers containing both λ DNA and the assay primer sequences (PTp1-IPC-F and PTp2-IPC-R; Table 2). This 617-bp product was then cloned into the TOPO vector (Invitrogen). Purified plasmid preparations were linearized and 0·005 pg linearized plasmid containing the λ/primer construct included in the PCR assay.

PCR amplification. Presence or absence of *B. pertussis* DNA in the respiratory samples was determined using two PCR assays, each specific for an independent region of the *B. pertussis* genome: (i) the pertussis toxin S1 (*ptxA*) promoter region (hereafter referred to as the *ptxA*-Pr PCR) and (ii) the insertion sequence IS*481* (hereafter referred to as the IS*481* PCR). Oligonucleotide primers targeting the *ptxA* promoter of *B. pertussis* (PTp1 and PTp2; Table 2) were used to amplify a 191-bp product (Houard *et al.*, 1989). Reaction mixtures were in a total volume of 50 µl and contained 2·5 mM $MgCl_2$, 15 mM Tris/HCl (pH 8·3), 50 mM KCl, 200 µM each deoxynucleotide (Roche Diagnostics), 20 pmol each primer (MWG Biotech) and 1·25 U AmpliTaq Gold (PE Applied Biosystems). Thermal cycling conditions used were a modification of those described by Mastrantonio *et al.* (1996): predenaturation for 10 min at 95 °C, and then 35 cycles consisting of denaturation for 15 s at 95 °C, annealing for 15 s at 66 °C and extension for 15 s at 72 °C, followed by a final extension for 5 min at 72 °C. Oligonucleotide primers targeting the insertion sequence IS*481* of *B. pertussis* (BP-1 and BP-2MOD; Table 2) were used to amplify a 145-bp product. Reaction mixtures were in a total volume of 50 µl and contained 2·5 mM $MgCl_2$, 10 mM Tris/HCl (pH 8·3), 50 mM KCl, 200 µM each deoxynucleotide (Roche Diagnostics), 20 pmol each primer (MWG Biotech) and 1·25 U AmpliTaq Gold (PE Applied Biosystems). Thermal cycling was carried out as follows: pre- denaturation for 10 min at 95 °C, followed by 35 cycles of denaturation for 15 s at 94 °C, annealing for 15 s at 56 °C and extension for 15 s at 72 °C, with a final extension for 5 min at 72 °C.

All extracted respiratory specimens were tested in PCR assays using 20 µl of undiluted extract, together with 1 : 10 and 1 : 100 dilutions (in nuclease-free water). Positive controls of purified *B. pertussis* DNA were included at three dilutions: (i) 1, 0·1 and 0·01 ng per reaction for the *ptxA*-Pr PCR and (ii) 0·1, 0·01 and 0·001 ng per reaction for the IS*481* PCR. Human DNA extracted from buccal cells was used as a positive control for the HMCO PCR assay at 100, 10 and 1 ng per reaction. Negative controls were included with each batch of samples tested and comprised one simulated sample of nuclease-free water (extracted as described above) with no added DNA. Amplification was performed using a Hybaid Thermocycler, a PTC-200 or a PTC-225 DNA Engine (MJ Research). The presence and size of amplification products were determined by agarose gel (2 % w/v; Life Technologies) electrophoresis in 1× Tris/borate/EDTA buffer. Gels were stained with 1 µg ethidium bromide ml^{-1} (Promega) and photographed under ultraviolet illumination (UV Transilluminator TS-40, Ultra-Violet Products) using Polaroid film 667 (Sigma).

Interpretation of PCR results. For the purposes of this study, PCR results for *B. pertussis* were divided into four classes: (i) 'consensus positive', defined by the observation of a band of the expected size in both *B. pertussis* PCR assays (191-bp amplicon in the *ptxA*-Pr PCR and 145-bp amplicon in the IS*481* PCR), following agarose gel electrophoresis and ethidium bromide staining; (ii) 'probable positive', defined by the repeatable observation of a band of the expected size in only one assay (*ptxA*-Pr or IS*481*) (i.e. seen in both the original and archive sample); (iii) 'doubtful', defined by the observation of a positive PCR result for only one target which was not repeatable and (iv) 'negative', where no PCR product was seen for either target.

ELISA for anti-pertussis toxin antibody in human sera. This assay was performed as described previously (Giammanco *et al.*, 2003a, b). The results are expressed as arbitrary enzyme units per millilitre ($eU\ ml^{-1}$). Pertussis toxin IgG antibody levels of greater than or equal to 100 $eU\ ml^{-1}$ were considered as evidence of recent *B. pertussis* infection (Nardone *et al.*, 2004). This cut-off value was taken for all serological specimens (acute, convalescent or single sera).

RESULTS AND DISCUSSION

Statutory notifications of pertussis in the UK are lower now than at any time since surveillance began (Crowcroft *et al.*, 2003). Despite this there is ample evidence that young infants

continue to develop pertussis and the associated mortality in this group is not insignificant (Crowcroft *et al.*, 2002). If intervention strategies are to be developed to address this problem it is essential that they are formulated on the basis of robust surveillance data. Practically this means seeking laboratory confirmation of putative cases rather than relying on clinical features alone. Laboratory confirmation by culture and isolation of the causative organism is generally considered to be the gold standard for the diagnosis of the majority of bacterial infections, because its specificity is effectively 100 %. *B. pertussis* is a fastidious and fragile organism and it is now widely recognized that reliance on culture alone is likely to lead to significant under-reporting of cases of pertussis infection (Müller *et al.*, 1997; Schmidt-Schläpfer *et al.*, 1997; Tilley *et al.*, 2000).

Although many serological and PCR assays have been developed to try to improve bacterial diagnosis, it has not always proved easy to interpret the data they yield. Whilst these assays might be expected to offer greater sensitivity than culture, they are unlikely to be 100 % specific. Consequently, the question arises, how does one differentiate between increased rates of positivity due to improved assay sensitivity (additional 'true' positives), against increased rates of positivity due to a decrease in assay specificity (false positives)? In their study of unrecognized pertussis in UK infants, Crowcroft *et al.* (2003) sought to maximize the overall diagnostic yield by using culture, serology and PCR. Further analysis of these data afforded us the opportunity to compare the diagnostic utility of each approach and to devise an optimal strategy for the laboratory diagnosis of pertussis.

Detection of *B. pertussis* by culture

Isolates were considered to be *B. pertussis* if colonies were Gram-negative, small coccobacilli, oxidase-positive, catalase-positive, showed agglutination with specific *B. pertussis* hyperimmune rabbit antisera and gave positive results by immunofluorescent microscopy using *B. pertussis*-specific mAbs. In this study a total of 356 respiratory samples from PICU infants (88), ward children (14) and contacts (254) were examined by culture (Table 1). Six samples (1·7 %; 95 % CI = 0·6–3·6) were culture-positive. Of these, *B. pertussis* was isolated from one (1·1 %; 95 % CI = 0–6·2) PICU infant, three (21·4 %; 95 % CI = 4·7–50·8) ward children and two (0·8 %; 95 % CI = 0·1–2·8) household contacts. Strenuous attempts were made to maximize the chances of recovering *B. pertussis* from clinical samples: specimens were collected and processed, by staff dedicated to the study, within 4 h of collection. Despite this, the combined isolation rate from the PICU infants and household contacts was less than 1 % (3 of 342). In the small group of ward children where the index of clinical suspicion was high, the isolation rate was much better, at 3 of 14 (21·4 %). Thus, the overall isolation rate obtained here of 1·7 % (6 of 356) probably represents a higher rate than would be seen in a routine diagnostic setting.

Detection of *B. pertussis* by PCR

We used an exhaustive three-stage approach to ensure the reliability of our PCR results. Our approach was: (i) to use a PCR assay for human DNA to determine the quality of the extracted samples and hence their suitability for analysis, (ii) to test for PCR inhibition and confirm assay integrity by using an IPC and (iii) to investigate suitable samples by consensus PCR, i.e. two independent targets on the *B. pertussis* genome.

Three samples (one from a child and two from adults) were considered unsuitable for analysis, as no amplification product was obtained using the primers targeting the HMCO gene with the original or archived sample. One sample was repeatedly inhibitory as indicated by the absence of an amplicon of expected size from the IPC. Results for the remaining 454 samples were considered valid. The presence or absence of human DNA in clinical specimens gives a good indication of the suitability of samples for testing by the species-specific PCR assays. Although we only found three samples to be unsuitable using this assay, we consider the use of the HMCO PCR to be beneficial in determining the suitability of samples for diagnostic PCRs, thus preventing false-negative results from being erroneously reported. In a clinical context further appropriate samples can be requested after consultation with the sending laboratory (Meade & Bollen, 1994).

Use of the IPC provided evidence of the integrity of each run and also identified the presence of PCR inhibitors in the samples. Failure of the PCR due to one or more of its components or to total inhibition is easily revealed by the absence of the IPC amplicon. Such failure would not be apparent without an IPC, again possibly leading to the erroneous reporting of false-negative results.

PCR sensitivity and specificity

The sensitivities of the *B. pertussis* PCR assays were determined to be approx. 1–10 organisms per reaction (10^3–10^4 ml^{-1}) for the *ptxA*-Pr PCR and approx. 0·1–1 organisms per reaction (10^2–10^3 ml^{-1}) for the IS*481* PCR, which is present in multiple copies per genome ($n = 238$, *B. pertussis* Tohama-I genome) (Parkhill *et al.*, 2003). The specificity of the PCR assays, which had previously been determined by lack of cross-reactivity with non-target organisms (Houard *et al.*, 1989; Glare *et al.*, 1990), was confirmed and extended in our laboratory. Only *B. pertussis* isolates gave bands of the expected size in the *ptxA*-Pr and IS*481* assays. This is despite the reported presence of IS*481*-like elements in both *B. holmesii* (Reischl *et al.*, 2001) and *B. bronchiseptica* (Gladbach *et al.*, 2002). Explanations for the lack cross-reactivity with these two species using our IS*481* PCR assay are: different sensitivities of the assays, the low and variable copy number of these elements in *B. holmesii* and *B. bronchiseptica* or the difference in the primer (probe) combination used and possible sequence variation in the target regions of these oligonucleotides.

Detection of *B. pertussis* DNA in respiratory specimens

Valid PCR results were obtained from 454 of 458 respiratory specimens. Of these, 105 (23·1 %; 95 % CI = 19·3–27·3) yielded at least one positive result with either of the two PCR targets, 52 (11·5 %) being either 'consensus' or 'probable' positive results. Detailed results are shown in Table 3. Of the single-target-only positive results, a positive in the IS*481* PCR accounted for all 14 of the 'probable' samples and 36 of the 'doubtful' samples, while a positive result in the *ptxA*-Pr accounted for the remaining 17 'doubtful' samples.

Comparison of PCR and culture

All six of the *B. pertussis* culture-positive samples were consensus positive by PCR, as were 13 culture-negative samples with PCR giving a more than threefold significantly higher diagnostic yield (McNemar's test; $P = 0{\cdot}0009$). If probable positive PCR results are taken into account, a further nine culture-negative samples were positive, with PCR giving an almost fivefold significant increase in diagnostic yield (McNemar's test; $P < 0{\cdot}0001$).

Anti-pertussis toxin antibody

Serological assays for the detection of *B. pertussis*-specific antibodies have been validated and used extensively in vaccine-related studies (Giammanco *et al.*, 2003a, b). However, they have not been used extensively in the UK for the diagnosis of infection. In this study we used an ELISA to determine anti-pertussis toxin IgG antibody levels. At least one serum sample was available from 331 of the study subjects. The distribution of these samples and the results obtained in relation to PCR testing are shown in detail in Table 4. Sera from 14/121 (11·6 %; 95 % CI = 6·5–18·7) eligible subjects were positive [9/111 (8·1 %; 95 % CI = 3·8–14·8) PICU infants; 5/10 (50 %; 95 % CI = 18·7–81·3) ward children, although a positive result ($\geqslant$100 eU ml^{-1}) was only seen in the convalescent sample for 8/10 subjects where both acute and convalescent sera were available]. Sera from 14/210 (6·7 %; 95 % CI = 3·7–10·9) household contacts were positive. The diagnostic yield obtained from serological evidence from the eligible subjects and household contacts was much higher than was obtained by culture. However, it should be noted that for the patient group (PICU infants and ward children) a diagnosis was usually only established where a convalescent sample was obtained. Thus, in a diagnostic context, serology was only useful in establishing a diagnosis retrospectively.

Comparison of PCR and serology

Overall serology and PCR results showed a high level of agreement (113/121; 94 %) for PICU infants and ward children, with 10/121 positive in both assays and 103/121 negative in both assays. Thus, there was no significant difference in yield between PCR and serology (McNemar's test, $\chi^2 = 0{\cdot}72$, $P < 0{\cdot}3$). However, 7/17 PCR consensus-positive subjects were not positive by serology, and conversely 4/14 serologically positive infants were not positive by PCR. One of the serologically positive cases gave a 'probable' positive PCR result. Complete correlation of PCR and serological data are unlikely, due to the different optimal (and actual) timing of specimen collection for both of these samples.

Nucleic acid detection has been used as a diagnostic tool in microbiology laboratories for almost a decade. To date, PCR remains the most widely implemented detection technology, because of its extreme sensitivity, specificity and diversity. However, despite these characteristics PCR remains a technically complex procedure and its successful implementation in a clinical setting is dependent on a number of factors including collection procedures, appropriateness and timing of sample, efficient extraction of intact target DNA, avoidance of contaminating DNA and removal of PCR inhibitors. The technical validity of a PCR assay must therefore be established before its clinical utility can be determined.

The use of two PCR assays targeting different regions on the *B. pertussis* genome allows considerable confidence to be placed on the results. Using this most stringent criterion, PCR was positive for 8·4 % of samples, a more than threefold increase in diagnostic yield than culture. This clearly illustrates the value of using PCR for the acute diagnosis of infants and children. If less stringent, but more typical, criteria were used for diagnosis by PCR (i.e. a repeatedly positive single target) then the diagnostic yield rose to 11·5 %, more than five times the diagnostic yield of culture. We would, however,

Table 3. Results of testing 454 samples by *B. pertussis* PCR

Subjects	Consensus positive (CP)			Probable positive (PP)			Combined CP+PP			Doubtful positive		
	n	%	95 % CI	*n*	%	95 % CI	*n*	%	95 % CI	*n*	%	95 % CI
Infants (122)	13	10·7	5·8–17·5	3	2·5	0·5–7·0	16	13·1	7·7–20·4	12	9·8	5·2–16·6
Ward (16)	5	31·3	11·0–58·7	1	6·3	0·2–30·2	6	37·5	15·2–64·6	2	12·5	1·6–33·3
Subtotal	**18**	**13·0**	**7·9–19·8**	**4**	**2·9**	**6·4–47·6**	**22**	**15·9**	**10·3–23·1**	**14**	**10·1**	**5·7–16·4**
Contacts (316)	20	6·3	3·9–9·6	10	3·2	1·5–5·7	30	9·5	6·5–13·2	39	12·3	8·9–16·5
Totals (454)	**38**	**8·4**	**6·0–11·3**	**14**	**3·1**	**1·7–5·1**	**52**	**11·5**	**8·7–14·7**	**53**	**11·7**	**8·9–15·0**

Table 4. Results of *B. pertussis* PCR on 458 subjects and pertussis toxin IgG assays where available

Serological specimen	Serological result	Consensus positive (*ptxA*-Pr and IS*481*)	Probable positive (*ptxA*-Pr or IS*481*)	Doubtful (*ptxA*-Pr or IS*481* on one occasion only)	PCR negative	Total
Infants (PICU)						
Acute and convalescent	Positive	5	1	0	2	8
	Negative	2	2	7	51	62
Acute only	Positive	1	0	0	0	1
	Negative	3	0	4	28	35
Convalescent only	Positive	0	0	0	0	0
	Negative	1	0	0	4	5
No. with sera		12	3	11	85	111
No. without sera		1	0	1	9	11
Total		**13**	**3**	**12**	**94**	**122**
Children (hospital ward)						
Acute and convalescent	Positive	0	0	0	1	1
	Negative	0	0	0	2	2
Acute only	Positive	1	0	0	0	1
	Negative	1	0	0	2	3
Convalescent only	Positive	3	0	0	0	3
	Negative	0	0	0	0	0
No. with sera		5	0	0	5	10
No. without sera		0	1	2	3	6
Total		**5**	**1**	**2**	**8**	**16**
Contacts						
Single serum	Positive	4	0	2	8	14
	Negative	9	6	25	156	196
No. with sera		13	6	27	164	210
No. without sera		7	4	12	83	106
Total		**20**	**10**	**39**	**247**	**320***
Overall Totals		**38**	**14**	**53**	**349**	**458**

*Four of these samples were inhibitory or unsuitable (see text).

caution against using a single target until considerable experience has been gained using it as part of a consensus PCR approach. A PCR assay may be thought to be specific for an organism when it is designed, but subsequently it may be found that its target gene is present in other closely related, or even unrelated, organisms. Although in this study no amplification was seen with purified DNA from *B. holmesii* or *B. bronchiseptica*, the presence of IS*481*-like sequences in these two additional *Bordetella* species potentially compromises the use of such assays in establishing the specific presence of *B. pertussis* (Reischl *et al.*, 2001; Gladbach *et al.*, 2002). However, it appears that the greater sensitivity of IS*481* assays (due to the presence of multiple copies) is a factor in their continued use by several laboratories. Moreover, as long as the cross-reactivity is acknowledged then, together with genuinely species-specific assays (such as the *ptxA*-Pr assay), the contribution of these other *Bordetella* species to the burden of respiratory disease may eventually be determined. We would thus recommend laboratories using only IS*481* assays to report positive results as 'evidence of *Bordetella* species' rather than 'evidence of *B. pertussis*'. Thus, we would prefer to consider such results (repeatably IS*481* PCR-positive only) as 'probably positive' for *Bordetella* species, and would seek confirmation of the diagnosis of *B. pertussis* infection by alternative means such as serology.

Frequently in published studies, a positive result for a single target is considered as evidence of infection, and no attempt is made to confirm this result by either repeating the assay or using a second PCR. These authors' view is that, in most situations, this is not a sufficiently rigorous approach. Of the 67 single-target positives in this study, 53 were not confirmed by repeat testing: 36 were positive by IS*481* and 17 by *ptxA*-Pr PCR (Table 3). In only two of the 27 patients for whom sera were also available did serology support the PCR result, casting considerable doubt on the validity of these 'doubtful' positive results. Explanation of these results is difficult, but they are most likely due to inter-sample contamination. Although processing a simulated 'negative' sample during

DNA extraction of each extracted batch of samples can reveal gross contamination, low-level inter-sample contamination is more difficult to control.

Concluding remarks

The use of consensus (two-target) PCR for *B. pertussis* enhances the specific rapid diagnosis of pertussis infection, particularly in infants (Crowcroft *et al.*, 2003), while serology has proved important for detection in older children and adults with cough of more than 2 weeks duration (Miller *et al.*, 2000). Whilst consensus PCR allows greater confidence to be placed on the combined test result, with the accumulation of experimental, epidemiological and clinical data and experience, single-target PCR-positive results may become acceptable. This consensus PCR approach can be readily transferred to 'real-time' PCR platforms allowing a more rapid turnaround and potentially increased sensitivity. However, for laboratories without access to such platforms, the consensus PCR approach for the detection of *B. pertussis* infection demonstrated here can be easily applied in a routine diagnostic laboratory setting with basic molecular biology equipment and reagents.

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