MOLECULAR DIAGNOSIS

Evaluation of a real-time PCR assay for detection of *Bordetella pertussis* and *B. parapertussis* in clinical samples

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A real-time PCR assay based on the TaqMan[®] technology was developed for the detection of *Bordetella pertussis* and *B. parapertussis* in clinical samples. The assay was evaluated with 182 specimens from 153 patients with and without symptoms of pertussis. The analytical sensitivity ranged from 0.1 to 10 cfu for *B. pertussis* and *B. parapertussis*, respectively, and diagnostic sensitivity was 94.1% when culture was used as a reference. No sample from a patient without symptoms of pertussis was positive in PCR. Twenty-four of 28 patients who were negative by culture and positive by PCR assay met the CDC clinical case definition for pertussis; the remaining four patients had paroxysms of shorter duration. Intra- and inter-assay variation were <5% and results were available within 4 h.

Introduction

PCR is being used increasingly in clinical microbiology laboratories for detection of *Bordetella pertussis* and *B. parapertussis* [1–3]. Various PCR protocols have been developed that target different regions of the genome, e.g., insertion sequences (IS) 481 and 1001 [1, 4–7], the pertussis toxin promoter region [4, 5, 8, 9], the adenylate cyclase gene [10] and the porin gene [11]. In a comparison of different PCR assays used in seven pertussis vaccine studies, the diagnostic sensitivity ranged from 73 to 100% when compared to culture, and false positive results were <1% [12].

PCR products are routinely detected and confirmed by gel electrophoresis or methods based on the annealing of specific oligonucleotide probes to the product. These methods generally require post-amplification handling of the PCR product, thereby increasing turn-round time as well as the possibility of PCR contamination. The TaqMan[®] technology obviates the need for post-amplification detection and confirmation by the use of a fluorescently labelled probe to detect, confirm and quantify the PCR product. During the extension phase of the PCR cycle, the 5′–3′ exonuclease activity of the Taq-polymerase cleaves the specifically hybridised fluorogenic probe, resulting in an increase of fluores-

cence emission. This assay format allows kinetic analysis of PCR product synthesis [13, 14]. In this study, a TaqMan[®] assay was developed for the detection of *B. pertussis* and *B. parapertussis* from nasopharyngeal swabs and aspirates and compared with conventional culture of clinical samples.

Materials and methods

Bacterial isolates and patient specimens

For optimisation of the PCR protocol, fresh isolates of *B. pertussis* and *B. parapertussis* were used. Colonies from Charcoal Agar (Oxoid, Wesel, Germany) containing defibrinated horse blood 10% and cephalexin 40 mg/L were suspended in phosphate-buffered saline (PBS) buffer to an optical density of 0.5 at 600 nm, corresponding to 1.0×10^9 cfu/ml for *B. pertussis* and *B. parapertussis* [15] and diluted in 10-fold steps. For preliminary comparison of TaqMan[®] PCR with culture, spiked swabs were produced by immersing dacron swabs (Hain Diagnostika, Nehren, Germany) into the different dilutions for 10 s. Swabs were held in Amies medium with charcoal (Hain Diagnostika) at room temperature for 48 h to simulate transportation.

As controls for determination of specificity, isolates of *B. bronchiseptica*, *B. trematum*, *Haemophilus influenzae*, β -haemolytic streptococci, *Corynebacterium* spp., *Staphylococcus epidermidis*, *Neisseria* spp., *Staph. aureus*, *Enterococcus* spp., *Streptococcus pneumoniae*, *Moraxella catarrhalis* and viridans streptococci were

Received 16 June 2000; revised version received 6 Oct. 2000; accepted 8 Nov. 2000.

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suspended in PBS to an OD₆₀₀ of 1.0. DNA extracted from *B. holmesii* ATCC 51541 (kindly donated by U. Reischl) was also used for specificity testing.

A total of 182 clinical samples (40 nasal swabs, 121 nasopharyngeal swabs, 12 nasopharyngeal aspirates, 8 pharyngeal swabs, 1 tracheal swab) was obtained from 153 inpatients and outpatients with or without symptoms of pertussis (74 male, 78 female), including one post-mortem sample (sex unknown). The patients ranged in age from 10 days to 75 years, with a mean of 62 months and a median of 22 months. Swabs were immersed in Amies medium with charcoal during transport; aspirates were sent without additives.

All specimens (spiked swabs and clinical samples) were plated on to charcoal agar, which was incubated at 37°C for 7 days. Swabs were then placed in 150 µl of PBS, swirled vigorously and squeezed out to elute any bacteria. If necessary, samples were stored at -20°C.

PCR

DNA was extracted with a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Primers and probes for PCR were designed by Primer Express software (PE Applied Biosystems, Weiterstadt, Germany) from the repeated insertion sequences IS481 (*B. pertussis*) and IS1001 (*B. parapertussis*) [16, 17]. The fluorescent reporter dye at the 5' end of the TaqMan probe was 6-carboxyfluorescein (FAM); the quencher dye at the 3' end was 6-carboxytetramethylrhodamine (TAMRA). Primers and probes are listed in Table 1. PCR was performed in two separate vials for *B. pertussis* and *B. parapertussis*; 5 µl of extracted DNA were added to 45 µl of a master mix. The final PCR reaction mix contained PCR buffer with ROX (6-carboxy-x-rhodamine) as a passive reference, 4 mM MgCl₂, 200 µM ATP, CTP and GTP, 400 µM UTP, AmpliTaq Gold polymerase 1.25 U, uracil-N-glycosylase 0.5 U, 900 nM primer PPert and APPert and 300 nM probe SPert, or 300 nM primer PParaP and APParaP and 300 nM probe SParaP, for detection of *B. pertussis* and *B. parapertussis*, respectively. All PCR reagents except ATP, CTP and GTP (Roche, Mannheim, Germany) were purchased from PE Applied Biosystems.

Amplification was performed in an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems) for 2 min at 50°C, 15 min at 95°C followed by 45 cycles of 15 s at 95°C and 1 min at 57°C. Results were analysed by Sequence Detection (SD) software (PE Applied Biosystems). For determination of specificity, amplification products of *B. pertussis*, *B. parapertussis* and other control strains were analysed on agarose 1.5% gels containing ethidium bromide 0.005%.

Results

PCR was optimised for each set of primers by adjusting MgCl₂, primer, probe and Taq polymerase concentration as well as cycle number and temperature. Increase of fluorescence intensity above a given threshold value as defined by SD software was scored as a positive result. A 'normalised reporter signal' is generated during each cycle by dividing the intensity of the reporter dye by the intensity of the passive reference. A positive signal is generated when the normalised reporter signal reproducibly exceeds the background fluorescence. The cycle in which the fluorescence intensity exceeds the threshold value is defined as threshold cycle (C_T). This cycle is dependent on the starting template copy number in a given PCR sample.

Analytical sensitivity was assessed by extraction and amplification of serial dilutions of suspensions of *B. pertussis* and *B. parapertussis* in PBS. The assay was able to detect 0.1–1 cfu and 1–10 cfu of *B. pertussis* and *B. parapertussis*, respectively, based on extraction of 1 ml of each dilution step. Both assays showed linearity over the whole range of eight 10-fold dilutions, as shown for *B. parapertussis* in Fig. 1. When spiked swabs were analysed by PCR and culture the TaqMan[®] assay improved sensitivity 10³–10⁴-fold for *B. pertussis* and *B. parapertussis*.

The primer and probe combination PParaP/APParaP/SParaP was able to amplify and detect *B. parapertussis* DNA specifically. With primer and probe combination PPert/APPert/SPert, positive signals were obtained for *B. pertussis* as well as for *B. holmesii*. DNA extracted from *Neisseria* spp. and *H. influenzae* showed some non-specific amplification products when analysed on agarose gel, but neither strain produced a positive signal with the TaqMan[®] PCR assay.

Table 1. Sequences of the oligonucleotide primers and probes

Oligonucleotide	Sequence (5' to 3')
<i>B. pertussis</i> – sense primer (PPert)	ATCAAGCACCGCTTACCC
<i>B. pertussis</i> – antisense primer (APPert)	TTGGGAGTTCTGGTAGGTGTG
<i>B. pertussis</i> – probe (SPert)	FAM -AATGGCAAGGCCGACGCTTCA- TAMRA
<i>B. parapertussis</i> – sense primer (PParaP)	GATATCAACGGGTGACGGATC
<i>B. parapertussis</i> – antisense primer (APParaP)	GTATGCCAACCAGTTTCGAA
<i>B. parapertussis</i> – probe (SParaP)	FAM -TGCTGCAATCGAGCAACGTGCA- TAMRA

Letters given in bold indicate fluorescent tags (see *Materials and methods*).

Fig. 1. Scatter plot of C_T against the number of cfu/ml of *B. parapertussis* in the bacterial suspensions used for DNA extraction and PCR assay. C_T is the threshold cycle, the PCR number at which the fluorescence intensity reaches a defined threshold value.

Intra-assay variation in 10 separate assays was found to be 1.4% and 4.0% for *B. pertussis* and *B. parapertussis*, respectively. Inter-assay variation of the same sample on five consecutive days was 3.8% and 4.9%, respectively.

Clinical samples were assayed in duplicate and, with discrepant results, PCRs were repeated in duplicate. Less than 5% of the results were discrepant – six specimens for *B. pertussis* and 10 for *B. parapertussis*. All except five samples were resolved by repeating the PCR. These five specimens were regarded as negative for further evaluation.

Of the total of 182 samples, 17 specimens (9.3%) from 14 patients were positive by conventional culture. Eleven specimens from nine patients yielded *B. pertussis*, five specimens from four patients gave *B. parapertussis*, one specimen gave both. PCR gave positive signals for 51 samples (28.0%) from 40 patients; 43 were positive for *B. pertussis* (33 patients), six for *B. parapertussis* (five patients) and two for both (two patients).

Of the 182 samples, 148 specimens were from 120 patients (56 male, 63 female, 1 sex unknown; age 1 month to 20 years), who presented with symptoms of pertussis. In this subpopulation, 45 specimens (30.4%) were positive for *B. pertussis* by PCR (35 of 120 patients), whereas only 12 samples (8.1%) were positive by culture (10 of 120 patients). Seven samples (4.7%) were positive for *B. parapertussis* by PCR (6 of 120 patients), and five (3.4%) samples were culture positive for *B. parapertussis* (4 of 120 patients) (Table 2). With PCR, two patients were positive for *B. pertussis* and *B. parapertussis*, whereas in culture only one sample showed a co-infection.

All culture-positive samples were considered true positives for PCR. One culture-positive sample was negative by PCR. Specimens positive by PCR only were resolved by review of patient history. Twenty-four of 28 patients who were culture-negative and PCR-positive met the CDC (Centers for Disease Control, USA) clinical case definition for pertussis (a cough illness lasting at least 2 weeks with paroxysms of coughing, inspiratory whoop or post-tussive vomiting).

Table 2. Sensitivity of real-time PCR for clinical specimens in relation to clinical symptoms

Clinical data	Number (%) of culture-positive samples	Organism detected	Number (%) of real-time PCR-positive samples
Paroxysms (n = 148)	12 (8.1)	<i>B. pertussis</i>	45 (30.4)
	5 (3.4)	<i>B. parapertussis</i>	7 (4.7)
No paroxysms (n = 34)	0	<i>B. pertussis</i>	0
	1	<i>B. parapertussis</i>	1*

*A 4-month-old girl with upper respiratory tract infection.

(<http://www.cdc.gov/nip/publications/sury-manual/pertussi.pdf>). The other four PCR-positive patients, although not meeting the CDC definition, had paroxysms of shorter duration.

The second subpopulation consisted of 34 specimens from 33 patients without clinical signs of pertussis (18 male, 15 female; age 10 days to 75 years). All except one sample were negative in PCR and culture. A swab from a 4-month-old girl yielded *B. parapertussis* and was positive for *B. parapertussis* by PCR. The patient's history showed an upper respiratory tract infection without paroxysms.

The diagnostic sensitivity of the TaqMan[®] assay was 94.1% when compared with culture. When compared with clinical symptoms, no sample from a patient without symptoms compatible with pertussis was positive in PCR, giving the assay a diagnostic specificity approaching 100%. The proportional increase of positive specimens by PCR – defined as PCR-positive culture-negative samples divided by the number of culture-positive samples – was 206%.

Discussion

This paper describes the evaluation of a real-time TaqMan[®] PCR assay for the detection of *B. pertussis* and *B. parapertussis* from nasopharyngeal swabs and aspirates. As described for other PCR systems [5, 6, 8, 9, 12, 18], the TaqMan[®] assay showed increased sensitivity over culture for detection of *B. pertussis* and *B. parapertussis*, and the detection rate of patients positive for *B. pertussis* or *B. parapertussis*, or both, was more than doubled. Similar increases in sensitivity through PCR have been observed in other published studies [1, 12]. At the same time, the assay seems highly specific. For all culture-negative, PCR-positive cases the clinical picture was compatible with a diagnosis of pertussis. It has to be kept in mind that *B. holmesii* also gave positive signals in the *B. pertussis* PCR, as demonstrated previously [19]. This appears to be due to the presence of IS481-like sequences in the *B. holmesii* genome (U. Reischl, personal communication). The clinical significance of this finding is not yet fully understood because, so far, *B. holmesii* has been isolated only infrequently from nasopharyngeal specimens [20, 21].

Reproducibility of the TaqMan[®] PCR was good, with intra- and inter-assay variation < 5%. Furthermore, the TaqMan[®] assay shows advantages over conventional PCR protocols. It eliminates the need for post-amplification handling, thereby reducing the risk of contaminating the PCR, and decreases the total analysis time to < 4 h. This is particularly helpful for rapid diagnosis of pertussis in children with life-threatening disease. TaqMan[®] PCR obviates gel electrophoresis with ethidium bromide and contact with hazardous

chemicals. It achieves high specificity and sensitivity by use of a specific probe, without the need for a nested PCR protocol. Real-time TaqMan[®] PCR might also be suitable for a duplex (*B. pertussis* and *B. parapertussis*) PCR, thereby increasing cost efficiency. Finally, it is well suited for quantification of genome copies, which has been advocated for research and routine pertussis diagnostics [7]. However, it is doubtful whether pre-analytic steps (e.g., obtaining nasopharyngeal swabs) can be standardised for quantification, and clinical implications of quantitative results have not been specified so far. In conclusion, real-time TaqMan[®] PCR offers a fast and economical tool with high sensitivity and specificity for the diagnosis of *B. pertussis* and *B. parapertussis* infections.

We thank Dr Udo Reischl for helpful discussions. This work was supported by a grant from the Bundesministerium für Bildung und Forschung (BMBF) (PID-ARI-Net).

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