A specific real-time PCR assay for the detection of *Bordetella pertussis*

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A novel real-time PCR (RT-PCR) assay was developed for detection of *Bordetella pertussis* in respiratory specimens by targeting the pertactin gene. *In vitro* evaluation with reference strains and quality control samples showed analytical sensitivity equivalent to and specificity superior to those of PCR assays which target the IS481 element. The pertactin-based RT-PCR assay offers better discrimination between *B. pertussis* and other *Bordetella* species than previously described assays.

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INTRODUCTION

Bordetella pertussis is the aetiological agent of whooping cough or pertussis. In the non-vaccinated population, it primarily affects children less than 6 months of age. The most common manifestations of *B. pertussis* infection are whooping cough and bronchitis with complications including pneumonia, seizures, encephalopathy and possibly sudden infant death (Mattoo & Cherry, 2005). During the last 15 years, the incidence of pertussis has increased again in different regions of the world despite intensive programmes of infant vaccination (Cordova *et al.*, 2000; De Melker *et al.*, 2000; De Schutter *et al.*, 2003; Gonzalez Moran *et al.*, 2002; Khetsuriani *et al.*, 2001). Adults and adolescents act as a reservoir for infection in very young infants who are not yet fully immunized and who experience severe morbidity. This reservoir exists because vaccination in childhood induces protection for a limited time only (Mattoo & Cherry, 2005; Von König *et al.*, 2002). The emergence and dissemination of *B. pertussis* variant strains that are antigenically different from vaccine strains is another possible cause of this phenomenon (Weber *et al.*, 2001).

In this context, rapid and accurate diagnostic tests for pertussis appear to be needed for improved management of cases and protection of infants. Most of the PCR-based methods described so far for the detection of *B. pertussis* target the multi-copy insertion sequence IS481 (Chan *et al.*, 2002; Cloud *et al.*, 2003; Poddar, 2003; Reischl *et al.*, 2001), which is also found in other *Bordetella* species (Muyldermans *et al.*, 2005). Here, we describe a novel PCR assay for specific detection of *B. pertussis* that we have designed by targeting the single-copy pertactin gene, a

69 kDa outer-membrane protein which is an important virulence factor of *B. pertussis*. The real-time pertactin PCR (pertactin RT-PCR) can be combined with sequence analysis of 16S rRNA genes for the identification of other species of *Bordetella*.

METHODS

Bacterial isolates. The assay specificity was evaluated on strains of *Bordetella* ($n=8$) and non-*Bordetella* species ($n=20$), including: *Bordetella pertussis* (ATCC 9340 and 9797), *Bordetella parapertussis*, *Bordetella bronchiseptica*, *Bordetella holmesii*, *Bordetella hinzii*, *Bordetella trematum*, *Bordetella avium*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus hominis*, *Streptococcus pneumoniae*, *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Morganella morganii*, *Enterobacter aerogenes*, *Citrobacter freundii*, *Proteus vulgaris*, *Burkholderia cepacia* (genomovar 1), *Escherichia coli*, *Moraxella catarrhalis*, *Serratia marcescens*, *Mycobacterium tuberculosis*, *Legionella pneumophila* and *Mycoplasma pneumoniae* (type 1 and 2).

All *Bordetella* strains were cultured on Regan–Lowe charcoal agar containing 10% defibrinated horse blood and cephalixin (40 ml l⁻¹) (Oxoid). Nucleic acids were extracted from one colony of fresh culture using the QiaAmp DNA mini kit (Qiagen), according to the manufacturer's instructions.

RT-PCR. The primers and probe for the real-time method (Fig. 1) were chosen by alignment of all described pertactin genes and determination of the consensus sequence using the Primer Express software (Applied Biosystems). The fragment to be amplified was checked by BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>) to avoid false-positive signals due to sequence homology. The TaqMan assays were performed on a GeneAmp 5700 (Applied Biosystems) in a 25 µl mix containing 12.5 µl TaqMan Universal Master Mix, which included optimized concentrations of AmpliTaq Gold DNA polymerase, AmpErase UNG, dNTPs with UTP, MgCl₂ and buffer (Applied Biosystems), 0.375 µl of each primer (300 nM) and 0.83 µl of the TaqMan probe (66 nM), purchased from MWG-Biotech AG,

Abbreviation: RT-PCR, real-time PCR.

	100	120	140
pm1	tggcgctggg	cgctggg	cgccgccccggcgccgcatgcccactggaacaaccagtcacatcgtc
pm2	tggcgctggg	cgctggg	cgccgccccggcgccgcatgcccactggaacaaccagtcacatcgtc
pm3	tggcgctggg	cgctggg	cgccgccccggcgccgcatgcccactggaacaaccagtcacatcgtc
pm4	tggcgctggg	cgctggg	cgccgccccggcgccgcatgcccactggaacaaccagtcacatcgtc
pm5	tggcgctggg	cgctggg	cgccgccccggcgccgcatgcccactggaacaaccagtcacatcgtc
pm6	tggcgctggg	cgctggg	cgccgccccggcgccgcatgcccactggaacaaccagtcacatcgtc
pm7	tggcgctggg	cgctggg	cgccgccccggcgccgcatgcccactggaacaaccagtcacatcgtc
pm8	tggcgctggg	cgctggg	cgccgccccggcgccgcatgcccactggaacaaccagtcacatcgtc
pm9	tggcgctggg	cgctggg	cgccgccccggcgccgcatgcccactggaacaaccagtcacatcgtc
pm10	tggcgctggg	cgctggg	cgccgccccggcgccgcatgcccactggaacaaccagtcacatcgtc
pm11	tggcgctggg	cgctggg	cgccgccccggcgccgcatgcccactggaacaaccagtcacatcgtc
Consensus TGGCGCTGGGCGCGCTGGGCGCGCCCCGGCGCGCATGCCGACTGGAACAACCAAGTCCATCGTC			
Primer Forward			
	160	180	200
pm1	aagaccgggtgagcgccagcatggcatccatccagggtccgacccggcgccgctacggaccgc		
pm2	aagaccgggtgagcgccagcatggcatccatccagggtccgacccggcgccgctacggaccgc		
pm3	aagaccgggtgagcgccagcatggcatccatccagggtccgacccggcgccgctacggaccgc		
pm4	aagaccgggtgagcgccagcatggcatccatccagggtccgacccggcgccgctacggaccgc		
pm5	aagaccgggtgagcgccagcatggcatccatccagggtccgacccggcgccgctacggaccgc		
pm6	aagaccgggtgagcgccagcatggcatccatccagggtccgacccggcgccgctacggaccgc		
pm7	aagaccgggtgagcgccagcatggcatccatccagggtccgacccggcgccgctacggaccgc		
pm8	aagaccgggtgagcgccagcatggcatccatccagggtccgacccggcgccgctacggaccgc		
pm9	aagaccgggtgagcgccagcatggcatccatccagggtccgacccggcgccgctacggaccgc		
pm10	aagaccgggtgagcgccagcatggcatccatccagggtccgacccggcgccgctacggaccgc		
pm11	aagaccgggtgagcgccagcatggcatccatccagggtccgacccggcgccgctacggaccgc		
Consensus AAGACCGGTGAGCGCCAGCATGGCATCCATATCCAGGGTCCGACCCGGCGCGCTACGGACCGC			
Probe(*) Primer reverse			

Fig. 1. Alignment of the 11 alleles of the *Bordetella pertussis* pertactin (*prn*) gene. (*) The probe used is modified 5'-FAM-AT-CGTCAGACCGGTGAGCGCC-TAMRA-3' (Taqman probe). Alignment positions were fixed following the complete sequence of the allele 1 pertactin gene. The accession numbers used for the alignment were: AJ011091 (*prn1*); AJ011092 (*prn2*); AJ011093 (*prn3*); AJ011015 (*prn4*); AJ011016 (*prn5*); AJ132095 (*prn6*); AJ133784 (*prn7*); AJ133245 (*prn8*); AJ315611 (*prn9*); AJ784875 (*prn10*); AJ507642 (*prn11*).

and 5 µl DNA template. An inhibitory control of each sample was performed using the TaqMan Exogenous Internal Positive Control kit (Applied Biosystems).

DNA quantification. Concentrations of *B. pertussis* extracted DNA were measured using the NanoDrop ND-1000 spectrophotometer. Tenfold serial dilutions were prepared in water using extracted DNA from three *B. pertussis* strains (ATCC 9340 and 9797 and Bord201). DNA dilutions ranging from 6 log₁₀ to 0.1 log₁₀ copy ml⁻¹ were used to determine the detection threshold of the assay.

DNA sequence analysis. To confirm the identification of *Bordetella* species, 16S rRNA gene PCR was performed using primers 16S rDNA forward [5'-T(A/T)ACACATGCAAGTCGA(A/G)CG-3' (nucleotide positions 22–42)] and 16S rDNA reverse [5'-CACCTGTGTTCCGG-TTCTCT-3' (nucleotide positions 1019–999)] (numberings deduced from *B. pertussis* strain Tohama AF366576) chosen using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). A single-round PCR was performed using a PCR mixture (50 µl) containing 25 µl Master Mix (Promega), 0.2 µM each primer and 5 µl target DNA. A first step of denaturation at 95 °C for 10 min was followed by 40 PCR cycles of 30 s at 94 °C, 1 min at 58 °C and 1 min at 72 °C and a final extension step for 7 min at 72 °C. The PCR products (998 bp) were sequenced using the BigDye Terminator kit (Applied Biosystems) according to the manufacturer's instructions. Each fragment obtained was analysed on the 3100 ABI sequencer (Applied Biosystems) and sequences were submitted to BLAST software for identification.

RESULTS AND DISCUSSION

The pertactin RT-PCR was evaluated with the strains listed in Methods and showed 100 % analytical specificity for *B. pertussis*. Two *B. pertussis* positive control strains and 10 *B. pertussis* pertactin variants typed with PFGE in a previous study (De Schutter *et al.*, 2003) were correctly identified by RT-PCR. All *Bordetella* species were correctly identified with the 16S rRNA gene sequence analysis with high degrees of similarity (over 98 %).

Standard curves were established with three strains of *B. pertussis*. The linearity of the assay was observed over a range of 6 log₁₀ of magnitude. The pertactin RT-PCR assay showed a limit of detection of 1 log₁₀ copies of *B. pertussis* DNA ml⁻¹. Inter-assay variability was tested by running the same controls with 10 replicates on different days; intra-assay variability was assessed using the three standard curves in five replicates for the same experiment. Using three *B. pertussis* strains, ATCC 9797, ATCC 9340 and Bord201, the detection rate of the assay was 90, 100 and 100 % at 10 log₁₀ copies ml⁻¹ of each strain. At 1 log₁₀ copies ml⁻¹, the detection rate fell to 50 % for strain ATCC 9797, 80 % for strain ATCC 9340 but still achieved 90 % for strain Bord201. The pertactin RT-PCR assay was highly reproducible with a coefficient of variation less than 5 % for inter-assay variability and less than 3 % for intra-assay variability.

We participated in two European external quality assessment ring trials for *B. pertussis* molecular detection (Muyldermans *et al.*, 2005). The first trial checked the sensitivity of protocols and the second one, containing *Bordetella non-pertussis*, tested the specificity. The analytical sensitivity of the pertactin RT-PCR varied from 9 to 30 c.f.u. ml⁻¹ whereas other laboratories using other PCR methods/target gene reported results ranging from 3 to 9000 c.f.u. ml⁻¹. The pertactin RT-PCR assay showed 100 % specificity while other participants' results varied in specificity from 73 to 87 %, mainly due to misidentification of *B. holmesii* and *B. bronchiseptica* (Muyldermans *et al.*, 2005).

RT-PCR is increasingly used in laboratory diagnostics. As compared with bacteriological culture, this technique is simple, fast and very sensitive (Dragsted *et al.*, 2004). However, the high sensitivity can lead to false-positive results, depending on the design of the assay. For *B. pertussis*,

the main target gene used is the insertion sequence IS481 (Chan *et al.*, 2002; Cloud *et al.*, 2003; Poddar, 2003; Reischl *et al.*, 2001), which is present in 50–100 copies. Insertion sequences are attractive because they occur in multiple copies, which increases the sensitivity of the PCR. However, IS481 is also present in *B. holmesii* and in *B. bronchiseptica* (Muyldermans *et al.*, 2005). In recent years, several reports showed pertussis-like infections that were attributed to *B. holmesii*; however, its clinical significance is not yet fully understood (Greig *et al.*, 2001; Mazengia *et al.*, 2000; Reischl *et al.*, 2001; Russell *et al.*, 2001). Insertion sequences of eubacteria are transposable elements that can provide sequence homologies at various sites on DNA molecules and they can result in DNA rearrangements and even in horizontal transfer (Arber, 2000; Mahillon & Chandler, 1998). Thus protocols using insertion sequences should be controlled by another region of the genome or another method, following previous recommendations (Fry *et al.*, 2004). The novel pertactin PCR assay described here showed an excellent analytical sensitivity for *B. pertussis* DNA detection compared to assays targeting insertion sequences. Furthermore, this novel assay alleviated the problems of cross-reactivity seen for other PCR strategies with other *Bordetella* species. Therefore, there was no requirement for a confirmation test. Although the pertactin gene exists in 11 known variants (Muyldermans *et al.*, 2004), the region targeted by the pertactin RT-PCR assay described here is conserved in all of them (Fig. 1). In summary, in the clinical laboratory, this novel RT-PCR assay appears promising for rapid diagnosis of pertussis. Awaiting further clinical validation, we recommend the use of the pertactin RT-PCR assay as a confirmatory test for specimens showing a positive result with an IS481-based assay.

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