Evaluation of amplification targets for the specific detection of *Bordetella pertussis* using real-time polymerase chain reaction

Mohammad Rubayet Hasan PhD1,2, Rusung Tan MD PhD FRCPC1,2,3, Ghada N Al-Rawahi MD FRCPC1,2, Eva Thomas MD PhD FRCPC1,2, Peter Tilley MD FRCPC1,2

BACKGROUND: *Bordetella pertussis* infections continue to be a major public health challenge in Canada. Polymerase chain reaction (PCR) assays to detect *B. pertussis* are typically based on the multicopy insertion sequence IS481, which offers high sensitivity but lacks species specificity.

METHODS: A novel *B. pertussis*-real-time PCR assay based on the porin gene was tested in parallel with several previously published assays that target genes such as IS481, ptx-promoter, pertactin and a putative thialase. The assays were evaluated using a reference panel of common respiratory bacteria including different *Bordetella* species and 107 clinical nasopharyngeal specimens. Discrepant results were confirmed by sequencing the PCR products.

RESULTS: Analytical sensitivity was highest for the assay targeting the IS481 element; however, the assay lacked specificity for *B. pertussis* in the reference panel and in the clinical samples. False-positive results were also observed with assays targeting the ptx-promoter and pertactin genes. A PCR assay based on the thialase gene was highly specific but failed to detect all reference strains of *B. pertussis*. However, a novel assay targeting the porin gene demonstrated high specificity for *B. pertussis* both in the reference panel and in clinical samples and, based on sequence-confirmed results, correctly predicted all *B. pertussis*-positive cases in clinical samples. According to Probit regression analysis, the 95% detection limit of the new assay was 4 colony forming units/reaction.

CONCLUSION: A novel porin assay for *B. pertussis* demonstrated superior performance and may be useful for improved molecular detection of *B. pertussis* in clinical specimens.

**Key Words:** Bordetella pertussis; IS481 element; Pertactin gene; Porin gene; Ptx-promoter; Real-time PCR

Despite high vaccine coverage, the number of reported cases of pertussis (whooping cough) has increased in recent years in the United States and Canada (1,2). Whooping cough is caused by the most widely known *Bordetella* species, *Bordetella pertussis*. For diagnosis, polymerase chain reaction (PCR)-based methods to detect *B. pertussis* in nasopharyngeal swabs or aspirates from patients with suspected pertussis are now commonly used due to their improved sensitivity and decreased turnaround time compared with bacterial culture (3). Currently, the most common PCR target is the multicopy insertion sequence IS481, which is repeated more than 200 times in the *B. pertussis* genome (4). IS481 PCR assays are widely used because of their high sensitivity. However, these assays lack specificity because the IS481 sequence is also present in the genomes of other *Bordetella* species (eg, *Bordetella bronchiseptica* and *Bordetella holmesii*) that are not generally associated with serious disease or widespread outbreaks (3,5-8). For example, a recent multicentre study conducted in 19 countries by the European surveillance network for vaccine-preventable diseases and the European Centre for Disease Prevention and Control show that misidentification of *Bordetella parapertussis* and *B. holmesii* as *B. pertussis* are common occurrences when using IS481-based PCR assays (9). These findings led to the conclusion that a positive sample from an IS481 assay indicates only the presence of *Bordetella* DNA and not the specific presence of *B. pertussis* DNA (9). The ability to detect *B. pertussis* with high specificity is important, not only because it allows for targeted therapy, but also because it could circumvent the implementation of infection control and public health measures, costly...
TABLE 1

Description of primers and probes used in the present study

| Assay     | Target gene        | Genome position | Primer/probe       | Sequence (5'–3')              | Working concentration | Reference |
|-----------|--------------------|-----------------|--------------------|-------------------------------|-----------------------|-----------|
| IS481     | Bordetella pertussis insertion sequence IS481 | 58491-58551† | Forward | CGGAACCGGATTGAGAACAC          | 330 nM                | 11        |
|           |                    | 58413-58432†    | Reverse            | TGGAAAGCTCACTGGGCAGC          | 330 nM                |           |
|           |                    | 58435-58456†    | Probe†             | CCGGCGGGGAAACCACCCCAATT      | 200 nM                |           |
| OMP       | B pertussis/B parapertussis gene for a porin protein | 868896-868918† | Forward | ATGCTATGGGGCGTGATCGG          | 240 nM                | 15        |
|           |                    | 365886-365888a‡ | Reverse‡           | TGGATGCGGGCGATCGGTC           | 240 nM                |           |
|           |                    | 869040-869066‡  | Probe‡             | TTGTTGTAAGTGGCAACATCTGGTC    | 240 nM                |           |
| BPTP      | B pertussis pertussis toxin gene, promoter region | 3988078-3988098 | Forward | TCTCGTATACAAAAACCTCTGGA      | 330 nM                | 12        |
|           |                    | 3988122-3988141 | Reverse§           | GTTCACTGGCACTGTTAGGTT         | 330 nM                |           |
|           |                    | 3988191-3988114 | Probe**            | CTCCGTCAGATC                  | 200 nM                |           |
| PRN       | B pertussis pertactin gene | 1090159-1098206 | Forward | TGGCGGATGAGAACACCA            | 330 nM                | 14        |
|           |                    | 1098243-1098261 | Reverse            | GTCGGAAGCCGCTAGATG            | 300 nM                |           |
|           |                    | 1098211-1098232 | Probe§             | ATCGTCAAGACCGGAGGCG           | 66 nM                 |           |
| BP283     | Putative thiolase gene (BP0026) | 30001-30040     | Forward            | CAGGCAACAGACGTTGCG            | 330 nM                | 13        |
|           |                    | 30104-30126     | Reverse            | GCCGAATACACCAAGTTGCA          | 330 nM                |           |
| PT-P      | B pertussis pertussis toxin gene, promoter region | 3988032-3988050 | Forward | CCATCCGGCACTACGTGG            | 330 nM                | Present   |
|           |                    | 3988108-3988127 | Reverse§           | GATTGCACTGGCCGAGT            | 330 nM                |           |
|           |                    | 3988090-3988098 | Probe**            | CGTCTGTAACAAACCTCTG          | 200 nM                |           |
| POR       | B pertussis gene for a porin protein (BPDT_0837) | 868978-869001† | Forward | TGAAATCGATGACACCTTTAA         | 330 nM                | Present   |
|           |                    | 869002-869046   | Reverse§           | CTTGCTCCCTTAATGCGGA           | 330 nM                |           |
|           |                    | 869003-869024   | Probe              | TCTCCACAGTATGACGC            | 200 nM                |           |

‡With respect to Bordetella pertussis Tohama I chromosome, complete genome (Accession No. NC_002929.2), unless otherwise specified; †Repeated many times (one position shown); $5′$-end labelled with 6-carboxyfluorescein (FAM) and 3′-end labelled with Black Hole Quencher 1 (BHQ1); ‡Primers also used for sequencing; §Primers also used for sequencing; **5′-end labelled with 6-carboxyfluorescein (FAM) and 3′-end labelled with MGB non-fluorescence quencher. BPTP Bordetella parapertussis toxin promoter; OMP Outer membrane porin protein; POR Porin; PRN Pertactin; PT-P Pertussis toxin promoter.

interventions that are generally unnecessary for nonpertussis Bordetella strains. Moreover, proper identification of pertussis cases enables more accurate assessment and enumeration of cases of vaccine failure – an issue of considerable public health importance.

Due to concerns about the false-positive results from IS481 PCR assays produced by nonpertussis Bordetella species, suggestions have been made to use multitarget PCR assays for definitive diagnosis of pertussis (10,11). These strategies, however, significantly increase workload, cost and turnaround time, and often involve complex interpretation rules before the results can be reported. A single-target PCR, carefully designed to specifically and exclusively amplify B pertussis DNA would, therefore, be preferable to current assays based on IS481 or multiple targets. Several real-time PCR assays designed to amplify non-IS481 single-gene targets have been described for the specific detection of B pertussis (12-14). In the present study, we evaluated the performance of these PCR assays in parallel with a novel real-time PCR assay that targets the B pertussis porin gene and the widely used IS481-based PCR assay.

METHODS

Bacterial strains and culture

The reference panel was comprised of B pertussis (American Type Culture Collection [ATCC] BAA-589, 9340 and 9797, and a clinical strain isolated at BC Children’s Hospital in Vancouver, British Columbia) from a patient nasopharyngeal aspirate), B parapertussis (ATCC 15237 and a clinical strain isolated at the BC Centre for Disease Control [Vancouver, British Columbia]), B holmesi (ATCC 51541), B bronchiseptica (ATCC BAA-588), Haemophilus influenzae (ATCC 10211), Pseudomonas aeruginosa (ATCC 27853), Klebsiella pneumoniae (ATCC 13882), Staphylococcus aureus (ATCC 43300), Staphylococcus epidermidis (ATCC 12228), Streptococcus agalactiae (ATCC 13813), Streptococcus mitis (ATCC 6249), Streptococcus pneumoniae (ATCC 49619), Streptococcus pyogenes (ATCC 9615), Corynebacterium pseudodiphtheritcum (ATCC 10700), Mycoplasma pneumoniae (ATCC 29342), Chlamydia pneumoniae (ATCC VR-1310) and a clinical strain of Neisseria mucosa isolated at BC Children’s Hospital. Bordetella species were streaked on charcoal agar medium (Oxoid) and grown at 37°C for 72 h in a humidified environment. Bacterial suspension was prepared in phosphate-buffered saline to a turbidity equivalent to a 0.5 McFarland standard, titred by colony counting and cryopreserved at −80°C. The titred bacterial stocks were diluted in TE8 buffer (10 mM Tris, 1 mM EDTA; pH 8.0) and used directly in the PCR reactions. Other bacteria were grown on blood or chocolate agar plates (Oxoid) overnight at 37°C in a 5% CO2 atmosphere, and a bacterial suspension was prepared in TE8 buffer to a turbidity equivalent to a 0.5 McFarland standard. The suspension was further diluted 1:10 in TE8 before being used for PCR.

Specimens

A total of 107 nasopharyngeal wash specimens were used in the present study, including selected PCR-positive (by a SYBR Green PCR [15]) specimens since August 2009, and specimens submitted between September 2011 to October 2012 to the Microbiology and Virology laboratory of BC Children’s Hospital for B pertussis PCR. Testing was performed exclusively on retrospective, residual samples that were stored at −80°C. To maintain patient anonymity, each sample was coded and all patient identifiers were removed to ensure that personnel involved in the present study were unaware of any patient information. Ethics approval was not considered to be necessary because studies that involve the secondary use of anonymous human biological materials are exempted from review by the local Research Ethics Board of the University of British Columbia. DNA from 0.2 mL nasopharyngeal wash specimens was extracted using the QIAamp DNA extraction platform QIAamp DNA Blood Maxi kit (Qiagen, USA).

PCR

The titred bacterial suspensions and DNA extracts from 107 nasopharyngeal wash specimens were analyzed using Taqman real-time PCR or by SYBR Green PCR with the primers and probes shown in Table 1. For Taqman real-time PCR assays, 5 μL of sample
Table 2: Specificity of different Bordetella pertussis polymerase chain reaction assays

| Sample                      | CFU/reaction | Published assays | In-house assays |
|-----------------------------|--------------|------------------|-----------------|
| **B. pertussis** (ATCC BAA-589) | 4.7×10³ | 22 15.9 22.6 24.3 24.5 | 33.4 24.9 |
| B pertussis (patient isolate) | 0.1×10³ | 27.2 19.8 29.6 27.6 31.1 | 37.9 31.8 |
| **B. parapertussis** (ATCC 15237) | 0.7×10³ | 22.1 34.7 0 0 0 | 0 0 |
| B parapertussis (patient isolate) | 0.1×10³ | 29.9 0 0 0 0 | 0 0 |
| **B. holmesii** (ATCC 51541) | 3.2×10³ | 28.9 18.3 0 0 0 | 0 0 |
| **B. bronchiseptica** (ATCC BAA-586) | 2.7×10³ | 23.2 33.5 33.1 0 39.6 | 0 0 |
| **Non-Bordetella species** |             |                  |                 |
| *Haemophilus influenzae* (ATCC 10211) | *0.05 M | 0 0 0 0 0 | 0 0 |
| *Pseudomonas aeruginosa* (ATCC 27853) | *0.05 M | 0 0 0 0 0 | 0 0 |
| *Klebsiella pneumoniae* (ATCC 13882) | *0.05 M | 0 0 0 0 0 | 0 0 |
| *Staphylococcus aureus* (ATCC 43300) | *0.05 M | 0 0 0 0 0 | 0 0 |
| *Staphylococcus epidermidis* (ATCC 12228) | *0.05 M | 0 0 0 0 0 | 0 0 |
| *Streptococcus agalactiae* (ATCC 13813) | *0.05 M | 0 0 0 0 0 | 0 0 |
| *Streptococcus mitis* (ATCC 6249) | *0.05 M | 35.4 0 0 0 0 | 0 0 |
| *Streptococcus pneumoniae* (ATCC 49619) | *0.05 M | 0 0 0 0 0 | 0 0 |
| *Streptococcus pyogenes* (ATCC 19615) | *0.05 M | 37.8 0 0 0 0 | 0 0 |
| *Corynebacterium pseudodiphtheriticum* (ATCC 10700) | *0.05 M | 0 0 0 0 0 | 0 0 |
| *Neisseria mucosa* (patient isolate) | *0.05 M | 0 0 0 0 0 | 0 0 |
| *Mycoplasma pneumoniae* (ATCC 29342) | 1CT=30 | 0 0 0 0 0 | 0 0 |
| *Chlamydophila pneumoniae* (ATCC VR-1310) | 1CT=31 | 0 0 0 0 0 | 0 0 |

*Sample concentration equivalent to 10-fold diluted, 0.5 McFarland standard; †Sample concentration equivalent to cycle threshold (CT) values according to respective real-time polymerase chain reaction assays. ATCC American Type Culture Collection; BPTD Bordetella parapertussis toxin promoter; CFU Colony forming units; M McFarland; OMP Outer membrane porin protein; POR Porin; PRN Pertactin; PT-P Pertussis toxin promoter*

**RESULTS**

Real-time PCR assays targeting the IS481 element (IS481), B pertussis/ B parapertussis gene for an outer membrane porin protein (OMP), pertussis toxin (ptx) promoter (BPTP), pertactin gene (PRN), and a putative thialase gene (BP283) (Table 1) (11-15) were selected from the literature. In addition, two new sets of primers and Taqman probes were designed based on ptx promoter (PT-P) and B pertussis gene for a porin protein (BPTD_0837) (POR). The new ptx promoter assay was designed through modification of the primer and probe sequences described by Grogan et al (12) in an attempt to further improve the specificity of the assay. By aligning the genomes of several different Bordetella species, it was noted that the published probe sequence has only a single nucleotide mismatch with the corresponding sequences of B bronchiseptica and B parapertussis genome. In contrast, the newly designed probe has >3 nucleotide differences with the corresponding sequences of B bronchiseptica and B parapertussis genome. The new POR assay was designed by exploiting the nucleotide sequence variation between the B pertussis gene for a porin protein and the corresponding sequences in the genomes of B parapertussis, B bronchiseptica and B holmesii (Figure 1).

All PCR assays were first tested using a panel comprised of ATCC strains and patient isolates of various Bordetella species and other bacteria commonly found in the respiratory tract (Table 2). The OMP extract or diluted bacterial suspensions was mixed with 20 μL of a master mix containing 12.5 μL of Taqman Universal PCR Master Mix (Applied Biosystems, USA) and primers and probes to final concentrations shown in Table 1. Thermal cycling was performed in a ABI7500 Fast instrument (Applied Biosystems, USA) with one cycle of 95°C for 10 min, followed by 45 cycles consisting of 95°C for 15 s and 60°C for 60 s. The SYBR Green PCR was performed with the same parameters except that the reaction mix contained 12.5 μL 2X Taq SYBR Green PCR Master mix (Bio-Rad, USA) and the thermal cycling was performed in a SmartCycler (Cepheid, USA) with one cycle of 95°C for 2 min, followed by 45 cycles consisting of 95°C for 10 s and 64°C for 60 s. The SYBR Green PCR reactions were subjected to meltcurve analysis after the final amplification cycle. Samples with discrepant results from different assays were analyzed by sequencing PCR products using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) in an ABI 3130 genetic analyzer according to the manufacturer’s instructions. For Probit regression analysis, B pertussis strain ATCC BAA-589 stock suspension was serially diluted to 10, 50, 100, 200, 500 and 1000 colony forming units (CFU)/mL, and eight replicates of each dilution were tested by real-time PCR; 95% detection limit was determined as described previously (16).
TABLE 3
Detection of *Bordetella pertussis* in nasopharyngeal samples from patients

| Sample | C<sub>q</sub> values from different assays | Sequencing |
|--------|----------------------------------------|------------|
| 1      | POR: 38.4, PRN: 38.3, BP283: 0, BPTP: 36.0, OMP: 36.0, IS481: 30.2 | *Pertussis* |
| 2      | POR: 37.5, PRN: 38.1, BP283: 0, BPTP: 34.5, OMP: 34.5, IS481: 31.6 | *Pertussis* |
| 3      | POR: 0, PRN: 0, BP283: 0, BPTP: 35.5, OMP: 35.5, IS481: 32.2 | *Bronchiseptica* |

Figure 2) Further analysis of discrepant samples. A Table showing parallel results of various PCR assays and sequencing for three discrepant samples (*Polymerase chain reaction [PCR] products that were sequenced*). B Alignment of sequences obtained from different PCR products with genome sequences from different *Bordetella* species (GenBank accession nos. HE965805 [*Bordetella pertussis*], BX640433 [*Bordetella parapertussis*], HE965806 [*Bordetella bronchiseptica*] and DQ420073 (*Bordetella holmesii*). Dots indicate homology with the derived sequences. For samples 2 and 3, only partial sequences were obtained because these PCR products are very small in size. For sample 3, two bases right after the forward primer (underlined) indicate that the sequence is obtained from *B* bronchiseptica genome. C<sub>q</sub> Cycle threshold; N Low-quality basecalls; OMP Outer membrane porin protein; POR Porin; PRN Pertactin

As a SYBR Green-based, shared primer assay designed to amplify both *B* pertussis and *B* parapertussis-specific DNA, but with different size PCR products that can subsequently be differentiated by their dissociation curves. When this PCR assay was applied to the panel of different *Bordetella* species and other respiratory bacteria, false-positive amplification was observed with *B* holmesii, *B* bronchiseptica, *S* suis and *S* pyogenes, but the dissociation curves were distinct from that of *B* pertussis and *B* parapertussis strains (Table 2). Apart from this assay, none of the other assays showed any cross-reactivity with other bacteria tested in the present study that do not belong to the genus *Bordetella*. The analytical sensitivity of IS481 assay for *B* pertussis was much higher (by cycle threshold >7) than other assays. The assay detected all *B* pertussis strains in the test panel, but also resulted in the false-positive detection of *B* parapertussis, *B* holmesii and *B* bronchiseptica strains, confirming the nonspecific nature of this target.

Among the single-target Taqman assays, the BPTP assay non-specifically detected *B* bronchiseptica along with *B* pertussis strains, while the ptx-promoter assay, PT-F, designed in the present study lacked sensitivity and failed to detect all *B* pertussis strains. The BP283 assay was specific to *B* pertussis but failed to detect two of the four *B* pertussis strains tested. The PRN assay detected all *B* pertussis strains but weakly detected *B* bronchiseptica at a relatively high concentration (≥5.4×10<sup>5</sup> CFU/mL). In contrast, the newly designed POR assay did not exhibit any cross-reactivity with DNA from any of the non-*B* pertussis strains.

Based on these results, the IS481, BPTP, BP283, PRN and POR assays were selected for further analysis of clinical samples. Among the 107 nasopharyngeal wash specimens, 21 (19.6%) samples were positive according to the IS481 assay, 20 (18.7%) samples were positive according to the BPTP and POR assays, and 19 (17.7%) samples were positive according to the BP283 and PRN assays (Table 3). Samples that produced discrepant results were further analyzed by sequencing the PCR products. There were three discrepant samples: a sample that was positive according to all assays except the BP283 assay; a sample that was missed by the PRN and BPTP assays; and a sample that was only positive using the BPTP and IS481 assays. Sequencing of PCR products confirmed that the first two samples were, in fact, *B* pertussis, while the third sample was *B* bronchiseptica (Figure 2). Taking into account the definitive sequence identification, the performances of all five *B* pertussis-specific assays were comparable, but the newly designed POR assay correctly detected all samples with a clinical sensitivity equal to that of IS481 assay. Based on a Probit regression analysis, the 95% detection limit of the POR assay was 4 CFU/reaction.

**DISCUSSION**

The diagnosis of whooping cough caused by *B* pertussis could be difficult, particularly at the early stages because the signs and symptoms of the disease closely resemble those of other common respiratory illnesses, such as those caused by *Mycoplasma pneumoniae*, *Corynebacterium*...
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B *pertussis*, also failed to detect one of the *B. pertussis*-positive clinical samples. The IS481 and BPTP assay, which detected a known ATCC strain of *B. bronchiseptica*, also produced one false-positive result among the clinical samples, which was eventually confirmed to be positive for *B. bronchiseptica* by sequencing. The results of using the PRN assay on the ATCC panel suggests that this assay could also falsely detect *B. bronchiseptica*, particularly at bacterial concentrations >10^5 CFU/mL in clinical samples. The novel real-time PCR assay that targets the porin gene of *B. pertussis* demonstrated superior performance over all other *B. pertussis* PCR assays tested in the present study.

The higher analytical sensitivity, but poor specificity of the IS481 PCR, is well described in the literature and is a generally accepted phenomenon (5-7,11). However, an independent comparison of the most common single-target PCR assays for *B. pertussis* has not been reported to date. Therefore, the results of the present study could be useful for guiding clinical microbiologists in the selection of an appropriate real-time PCR assay for *B. pertussis*. The new PCR gene-based PCR assay described may serve as a valuable new tool for diagnostic laboratories seeking to improve the specificity of *B. pertussis* detection, with sensitivity comparable with that of IS481 PCR assays.

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