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Identification and Evaluation of New Target Sequences for Specific Detection of *Bordetella pertussis* by Real-Time PCR▽

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A comparative analysis of the *Bordetella pertussis*, *B. bronchiseptica*, and *B. parapertussis* genome assemblies permitted the identification of regions with significant sequence divergence and the design of two new real-time PCR assays, BP283 and BP485, for the specific detection of *B. pertussis*. The performance characteristics of these two assays were evaluated and compared to those of culture and an existing real-time PCR assay targeting the repetitive element IS*481*. The testing of 324 nasopharyngeal specimens indicated that, compared to culture, the BP283 assay had a sensitivity and specificity of 100 and 96.8% and the BP485 assay had a sensitivity and specificity of 92.3 and 97.1%. Notably, *B. holmesii* was isolated from two specimens that were positive by the IS*481* assay but negative by the BP283 and BP485 assays. These two assays represent an improvement in specificity over those of PCR assays targeting only IS*481* and may be duplexed or used in conjunction with existing PCR assays to improve the molecular detection of *B. pertussis*.

Bordetella pertussis, the causative agent of pertussis, or whooping cough, is reemerging as a significant respiratory pathogen in many parts of the world (2, 14, 26). In the United States, the number of pertussis cases has increased rapidly over the last several years (2). Explanations for this reemergence include waning herd immunity, the antigenic drift of *B. pertussis* strains away from the antigenic composition of vaccines, better pertussis awareness and reporting, and the increasing use of nucleic acid amplification tests for the laboratory detection of *B. pertussis* (3, 16). Nucleic acid amplification tests, such as the PCR test, offer an improvement in sensitivity over that of culture for the diagnosis of pertussis (23). Unfortunately, there is no standardized PCR test available for *B. pertussis* detection by clinical microbiology laboratories. Among the previously described PCR assays for pertussis diagnosis, the most frequently utilized target sequence is that of IS*481*. Over 200 copies of this insertion element are found in the *B. pertussis* genome (18). This high copy number offers a significant advantage in analytical sensitivity for PCR assays utilizing this target compared to assays with single-copy target sequences (23). However, the enhanced sensitivities of assays targeting IS*481* also make them more prone to give false-positive results through laboratory contamination. Moreover, assays detecting the IS*481* target sequence also lack specificity, as IS*481* elements are also found in *B. holmesii* and some *B. bronchiseptica* strains (21, 22). Reports of respiratory disease outbreaks attributed to *B. pertussis* based on errant results of PCR tests targeting IS*481* have prompted recommendations for the use of additional PCR targets for the confirmation of *B. pertussis* detection by PCR (2, 5, 7, 11, 19, 20).

In this study, we describe the identification of two new PCR target sequences through comparative genomics. Real-time PCR assays targeting these sequences were developed in simplex and duplex formats, and their performances were compared to those of culture and an IS*481* real-time PCR assay (10) for the detection of *B. pertussis* in clinical specimens.

MATERIALS AND METHODS

Strains. The phenotypic identification of *B. pertussis* isolates was based on oxidase activity, the lack of motility and urease activity, the inability to reduce nitrate, and the results of confirmatory testing by slide agglutination or direct fluorescent-antibody testing. *B. holmesii* isolates were differentiated from other *Bordetella* spp. based on the lack of oxidase and urease activity, the production of a soluble brown pigment, and the absence of motility, and the identification was confirmed by cellular fatty acid analysis. Organisms utilized in specificity testing were clinical isolates identified at the California Department of Public Health by standard methods.

Specimens. Specimens for pertussis testing were collected in two Northern California counties, Alameda and Yolo, over a 5-year period from 2003 to 2007. Specimens were submitted for public health surveillance from individuals having any of the following: (i) symptoms consistent with the CDC clinical case definition of a cough illness lasting longer than 2 weeks with paroxysms of coughing, inspiratory whoop, and/or posttussive vomiting; (ii) for individuals with exposure to a patient whose illness met the aforementioned clinical case definition, an acute cough illness; or (iii) for infants less than 3 months of age, a respiratory illness with apnea. Clinical data on individuals whose specimens were submitted for testing were not systematically collected and, therefore, were not incorporated into this study.

The Alameda and Yolo County public health laboratories (PHL) performed culture analyses of patient samples collected within their respective jurisdictions, whereas PCR was performed by the California Department of Public Health's Microbial Diseases Laboratory. The Alameda County PHL collected two nasopharyngeal specimens from each patient by using Dacron swabs and planted each swab into Regan-Lowe transport medium. One swab was utilized for culture isolation, and the other was submitted for PCR testing. Up until September 2007, patient samples collected by the Alameda County PHL were plated onto Bordet-Gengou medium with methicillin, Regan-Lowe medium without antibiotics, and brain heart infusion medium. Thereafter, the Bordet-Gengou medium was replaced with Regan-Lowe with cephalexin. The Yolo County PHL collected one nasopharyngeal specimen per patient by using a Dacron swab and placed the swab into Regan-Lowe transport medium. The swab was used to inoculate

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TABLE 1. Primer and probe sequences

PCR target	Forward primer	Reverse primer	Probe[a]
BP283 region	CAGGCACAGCACGTATTGCG	GACGATTACCAGCGAGATTACGA	CCGCCATCGCAACCGTCGCATTCA
BP485 region	CGAGCCACTGTTTCTATTGATTGA	CGGGCCTCATCTTCGTTCAG	TGTGCGTGTTTTCCCCAGAGCCCC
IS*481*[b]	ATCAAGCACCGCTTTACCC	TTGGGAGTTCTGGTAGGTGTG	AATGGCAAGGCCGAACGCTTCA

[a] Hydrolysis probes were dually labeled with the reporter fluorophore FAM and Black Hole Quencher 1 or the reporter fluorophore Pulsar 650 and Black Hole Quencher 2.
[b] Primer and probe sequences are given in reference 10.

Regan-Lowe medium with cephalexin, as well as a chocolate agar plate. After medium inoculation, the swab was submitted for PCR analysis. For both laboratories, cultures were incubated at 35°C for 7 to 12 days and monitored for the presence of suspicious colonies. Confirmatory testing for *B. pertussis* was performed as described above.

Nucleic acid extracts. Nucleic acids from bacterial isolates and fungal cultures were extracted using a heat lysis procedure. A partial 1-μl loopful of growth was resuspended in 100 μl of 10 mM Tris, pH 8.0, and heated for 5 min at 95°C. The lysate was then centrifuged for 5 min at 13,000 × *g*, and the supernatant was removed for use as a nucleic acid extract for PCR.

Purified nucleic acids were extracted from nasopharyngeal specimens. Each nasopharyngeal swab was immersed in 0.4 ml of ATL buffer (Qiagen Incorporated, Valencia, CA) and subjected to a vortex intermittently over a 15-min period to release collected material, and then approximately half of this material was used for the extraction of nucleic acids. In the initial stages of this project, specimens were processed using a manual nucleic acid extraction procedure. This procedure was then replaced by a semiautomated nucleic acid extraction method. In side-by-side trials with spiked material, the two methods had very similar efficiencies for the recovery of *B. pertussis* DNA. For manual nucleic acid extraction, the DNeasy tissue kit was used according to the recommendations of the manufacturer (Qiagen Incorporated). The semiautomated procedure utilized the MagNA Pure Compact instrument (Roche Applied Science, Indianapolis, IN) and prefilled reagent cartridges supplied as MagNA Pure Compact nucleic acid isolation kit 1. An external lysis step in which 0.17 ml of the swab eluent was mixed with 0.21 ml of a bacterial lysis buffer (Roche Applied Science) and 0.02 ml of 20-mg/ml proteinase K (Roche Applied Science) was performed. Afterward, the samples were incubated for 10 min at 56°C and then for 10 min at 95°C before being loaded onto the instrument to complete the extraction. The instrument was run using the total nucleic acid plasma external lysis program. A low-copy-number negative control (consisting of *B. bronchiseptica* whole organisms) and a low-copy-number positive control (consisting of *B. pertussis* nucleic acid) were included in each run. A negative control without any analyte was added for every five clinical specimens tested.

PCR. Primers and probes were synthesized by BioSearch Technologies (Novato, CA), and the sequences are shown in Table 1. Primer and probe sets corresponding to the newly identified PCR target regions were designated BP283 and BP485. Dually labeled hydrolysis probes were generated using the reporter fluorophore 6-carboxyfluorescein (FAM) or Pulsar 650 with Black Hole Quencher 1 or Black Hole Quencher 2, respectively.

Real-time PCR was performed on a LightCycler 1.2 instrument (Roche Applied Sciences) using the following amplification parameters: one step of 50°C for 2 min, followed by one step of 95°C for 10 min and 45 cycles of 95°C for 10 s and 60°C for 30 s. All real-time PCR assay mixtures contained 800 nM (each) primers, 0.5 U of heat-labile uracil-DNA glycosylase, and 1× LightCycler Fast-Start DNA Master HybProbe (Roche Applied Science). The $MgCl_2$ concentrations for the BP283, BP485, and IS*481* assays were 2.5, 4, and 4 mM, and the probe concentrations were 200, 100, and 200 nM, respectively. For the duplex real-time PCR assay, the reaction mixture contained 4 mM $MgCl_2$, 200 nM FAM-labeled BP485 probe, and 600 nM Pulsar 650-labeled BP283 probe. Five microliters of nucleic acid extract was used in a final reaction volume of 20 μl. Cycle threshold (C_T) values were determined using the LightCycler's Fit Points analysis software. Any C_T value was considered to be a positive test result. Each specimen was tested in a separate reaction for the presence of inhibitory substances by real-time PCR assays targeting either human RNase P or beta-actin genes as heterologous controls.

RESULTS

Identification of target sequences. A comparative sequence analysis was performed in August 2001 using the draft genome assemblies for *B. pertussis* strain Tohama I, *B. parapertussis* strain 12822, and *B. bronchiseptica* strain RB50 available through the National Center for Biotechnology Information. In an effort to identify areas with significant sequence divergence, approximately 1 Mbp of the *B. pertussis* genome was investigated by performing genomic BLAST searches of the *B. parapertussis* and *B. bronchiseptica* genomes with sequential 10-kbp segments of the *B. pertussis* genome. Excluding insertion elements, two genomic segments were found to have significant sequence divergence from corresponding regions of the *B. parapertussis* and *B. bronchiseptica* genomes. A segment of approximately 3.8 kbp consisting of nucleotides 28315 to 32100 was unique to the *B. pertussis* genome (nucleotide numbering and locus tags are derived from the finished genome sequence [GenBank accession number BX470248]) and was used to design the primer and probe set designated BP283. The BP283 primers amplify nucleotides 30021 to 30126, located within a putative thiolase gene tagged BP0026. A second region delineated by nucleotides 428500 to 428700 displayed significant sequence divergence from the corresponding regions of the *B. parapertussis* and *B. bronchiseptica* genomes and provided a target sequence for the second primer and probe set, referred to as BP485. These primers amplify nucleotides 428551 through 428668, located between BP0426 (*cspA*) and BP0427 (a hypothetical-protein gene).

Evaluation of the BP283 and BP485 real-time PCR assays. Dually labeled hydrolysis probes were utilized to permit real-time PCR detection by the 5′ exonuclease assay. Following the optimization of reagent concentrations, the performance characteristics of the BP283 and BP485 real-time PCR assays were determined and compared to those of a similarly formatted assay targeting IS*481*. A panel of *Bordetella* and non-*Bordetella* strains was tested to evaluate the specificity of each assay. The results of this evaluation are provided in Table 2. The BP283 and BP485 assays were specific for *B. pertussis*. As expected, the IS*481* assay detected both *B. pertussis* and *B. holmesii*. IS*481* target sequences have also been identified in some strains of *B. bronchiseptica* (21, 25). However, none of the five *B. bronchiseptica* strains tested in our study were detected by the IS*481* assay. The limit of detection for each assay was determined by testing serial 10-fold dilutions of a suspension of enumerated *B. pertussis* CFU. Following nucleic acid extraction and real-time PCR, the detection limits for the BP283, BP485, and IS*481* assays were found to be 1.0, 1.0, and 0.1 CFU/reaction, respectively. As described in earlier studies, the observed lower detection limit of the IS*481* assay likely reflects the availability of multiple copies of the IS*481* target sequence in the *B. pertussis* genome (9, 10, 22). Standard curves generated from the performance data indicated that the amplifica-

TABLE 2. Specificities of PCR targets

Organism	No. of strains tested	No. of strains positive by PCR for:		
		IS*481*	BP485 region	BP283 region
B. pertussis	30	30	30	30
B. bronchiseptica	5	0	0	0
B. hinzii	1	0	0	0
B. holmesii	10	10	0	0
B. parapertussis	5	0	0	0
Non-*Bordetella* strains[a]	34	0	0	0

[a] Non-*Bordetella* strains included strains of *Achromobacter xylosoxidans*, *Actinomyces israelii*, *Actinomyces naeslundii*, *Bacillus anthracis*, *Burkholderia cepacia*, *Burkholderia pseudomallei*, *Chlamydia pneumoniae*, *Coccidioides immitis*, *Corynebacterium diphtheriae*, *Cupriavidus pauculus*, *Eikenella corrodens*, *Escherichia coli*, *Finegoldia magna*, *Francisella tularensis*, *Haemophilus aphrophilus*, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Klebsiella pneumoniae*, *Klebsiella rhinoscleromatis*, *Legionella anisa*, *Legionella pneumophila*, *Moraxella catarrhalis*, *Mycobacterium tuberculosis*, *Mycoplasma pneumoniae*, *Neisseria meningitidis*, *Nocardia brasiliensis*, *Nocardia asteroides*, *Pasteurella multocida*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Yersinia pestis*.

TABLE 3. Performance characteristics of PCR assays for the detection of *B. pertussis* in clinical specimens

Assay	No. of positives[a]	Sensitivity (%)	Specificity (%)	PPV[b] (%)	NPV[c] (%)
Culture	13	NA[d]	NA	NA	NA
BP485 PCR	22	92.3	97.1	57.1	99.7
BP283 PCR	23	100	96.8	56.5	100
IS*481* PCR	42	100	90.6	31.0	100

[a] Number of the 324 nasopharyngeal specimens tested yielding positive results.
[b] PPV, positive predictive value. PPV = (number of true-positive results/number of true-positive results plus number of false-positive results) × 100.
[c] NPV, negative predictive value. NPV = (number of true-negative results/number of true-negative results plus number of false-negative results) × 100.
[d] NA, not applicable (reference method).

tion efficiencies (efficiency = $10^{1/\text{slope}} - 1$) of the BP283, BP485, and IS*481* PCR assays were 0.923, 1.05, and 0.937, respectively. The reproducibility of results was measured by testing low and moderate concentrations of *B. pertussis* cells in replicates of four on three separate occasions and was found to be 100% for all three assays.

Of the 324 nasopharyngeal specimens tested, 13 were positive by culture. Twenty-two, 23, and 42 specimens were positive by the BP485, BP283, and IS*481* assays, respectively. The inhibition of PCR was observed for three specimens. The performance characteristics and predictive values of the PCR assays are summarized in Table 3. Compared to culture, the BP485 assay had a sensitivity and specificity of 92.3 and 97.1%, respectively, and the BP283 assay had a sensitivity and specificity of 100 and 96.8%, respectively. The BP485 assay failed to detect *B. pertussis* in 1 of 13 culture-positive specimens. Repeat testing of this one specimen in triplicate by the BP485 assay produced a positive result for two of the replicates. This specimen also yielded high C_T values in the IS*481* and BP283 assays, suggesting the presence of limiting amounts of target nucleic acids or inefficient nucleic acid recovery. The BP485 and BP283 assays detected the presence of *B. pertussis* in 10 culture-negative specimens. All of these specimens were also positive by the IS*481* assay. The IS*481* assay had a sensitivity and specificity of 100 and 90.6%, respectively, compared to culture. Nineteen specimens that were negative for *B. pertussis* by culture and both the BP485 and BP283 assays were positive by the IS*481* assay. Significantly, *B. holmesii* was isolated from two of these IS*481* assay-positive specimens. Of the 17 remaining specimens, all yielded C_T values greater than 33.6 when tested by the IS*481* assay, suggesting the presence of limiting amounts of the target sequence in these specimens.

Duplex real-time PCR using the BP283 and BP485 targets. The primer and probe sequences for the BP283 and BP485 assays were designed to minimize interactions with one another and facilitate the duplexing of oligonucleotides into a single PCR assay. To permit the duplexing of hydrolysis probes on the LightCycler instrument, model 1.2, one probe was labeled with the reporter fluorophore FAM and the other probe was labeled with the reporter fluorophore Pulsar 650. In this duplex format, the detection limit (1 CFU/reaction) was identical to that for either PCR target tested individually. When evaluated retrospectively against a panel of 12 culture- and PCR-positive samples and 20 culture- and PCR-negative samples, the duplex real-time PCR assay was 100% accurate.

DISCUSSION

The determination of the genome sequences of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* was completed and the sequences were reported in 2003 by Parkhill et al. (18). We utilized early (August 2001) assemblies of these genome sequences to search for PCR target sequences that might be specific to *B. pertussis*. Two regions with significant sequence divergence were identified and utilized for the design of the BP283 and BP485 real-time PCR assays. Notably, the diagnostic potential of the region used for the design of our BP485 assay was also predicted previously by others using microarray-based comparative genome hybridization (4). Similarly, previous work using representational difference analysis described the genome region encompassing the BP283 target sequence as being specific to *B. pertussis* (15). Both assays demonstrated excellent sensitivities and specificities when applied to clinical isolates and nasopharyngeal specimens. In contrast to the IS*481* assay, the BP283 and BP485 assays detected none of the 10 *B. holmesii* strains tested. The detection limit for both the BP283 and BP485 assays was 1 CFU per reaction, which is comparable to those of other PCR assays based on single-copy PCR targets. However, the BP283 and BP485 assays were at least 10-fold less sensitive than the IS*481* assay, which targets multiple copies of the repetitive IS*481* element. Given the difference in the limits of detection, discordant results based on these single- and multiple-copy PCR targets would be expected in the testing of specimens having limiting amounts of *B. pertussis* DNA. Indeed, this scenario appeared to be the case when these three assays were applied to our panel of 324 nasopharyngeal specimens. There were discordant results for 19 specimens, which were positive by the IS*481* assay but negative by the BP283 and BP485 assays. Eighteen of these samples had high C_T values (>33.6) by the IS*481* assay, suggesting either that the amounts of *B. pertussis* DNA present were beyond the detection limit of the BP283 and BP485 assays or that the IS*481* signal was associated with DNA from another

Bordetella species. Indeed, *B. holmesii* was recovered from 2 of the 19 samples with discordant PCR results. One of these *B. holmesii*-positive samples yielded a low C_T value (28 cycles) when tested by the IS*481* assay, indicating relatively large amounts of *B. holmesii* DNA.

The ability of *B. holmesii* to confound the identification of *B. pertussis* when the IS*481* target sequence is used has been the subject of much previous discussion (12, 17, 22, 23). However, the isolation of *B. holmesii* following a positive IS*481* PCR result has been alluded to in only a single report (24), and *B. holmesii* has not appeared to be a source of false-positive results in other studies. A large study using *B. holmesii*-specific PCR assays failed to detect *B. holmesii* in respiratory specimens from Finnish and Dutch patients (1). Similar findings for a patient population in Alberta, Canada, have been described previously (9). Although our sample size was small, the frequency of *B. holmesii* isolation (0.6%) from nasopharyngeal specimens in our study is similar to that described in an earlier study by the Massachusetts Department of Public Health (27). While it is tempting to speculate that like *B. parapertussis*, *B. holmesii* may be associated with a milder pertussis-like illness, the clinical relevance of isolating *B. holmesii* from the upper respiratory tract remains to be established (27).

The lack of specificity associated with the IS*481* target sequence has led some groups to recommend the use of alternate target sequences or multiple PCR targets for the confirmation of *B. pertussis* detection. Among the various target sequences evaluated, only the pertussis toxin promoter and the porin gene have been shown to be specific for *B. pertussis* (6, 8, 13). Our study provides two new target sequences that can be used in combination with existing PCR targets or duplexed to improve the accuracy of *B. pertussis* detection for patient diagnosis and outbreak recognition.

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