

Novel Multitarget Real-Time PCR Assay for Rapid Detection of *Bordetella* Species in Clinical Specimens[∇]

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A novel multitarget real-time PCR (RT-PCR) assay for the rapid identification of *Bordetella pertussis*, *B. parapertussis*, and *B. holmesii* was developed using multicopy insertion sequences (ISs) in combination with the pertussis toxin subunit S1 (*ptxS1*) singleplex assay. The RT-PCR targets for the multiplex assay include IS481, commonly found in *B. pertussis* and *B. holmesii*; IS1001 of *B. parapertussis*; and the IS1001-like sequence of *B. holmesii*. Overall, 402 *Bordetella* species and 66 non-*Bordetella* species isolates were tested in the multitarget assay. Cross-reactivity was found only with 5 *B. bronchiseptica* isolates, which were positive with IS1001 of *B. parapertussis*. The lower limit of detection (LLOD) of the multiplex assay was similar to the LLOD of each target in an individual assay format, which was approximately 1 genomic equivalent per reaction for all targets. A total of 197 human clinical specimens obtained during cough-illness outbreak investigations were used to evaluate the multitarget RT-PCR assay. The multiplex assay results from 87 clinical specimens were compared to the individual RT-PCR assay and culture results. The multitarget assay is useful as a diagnostic tool to confirm *B. pertussis* infections and to rapidly identify other *Bordetella* species. In conclusion, the use of this multitarget RT-PCR approach increases specificity, while it decreases the amount of time, reagents, and specimen necessary for RT-PCRs used for accurate diagnosis of pertussis-like illness.

Pertussis, an acute respiratory infection caused by *Bordetella pertussis*, continues to be a significant cause of morbidity and infant mortality worldwide (31). Several factors have been attributed to an increasing incidence of pertussis, including waning immunity, increased public health awareness and reporting, and use of more sensitive diagnostics such as the PCR assay. Among several chromosomal regions utilized for real-time PCR (RT-PCR) detection of *B. pertussis*, the multicopy insertion sequence (IS) IS481 (8) is often the target of choice because it is found in multiple copies in *B. pertussis* (50 to 238 copies per genome) (19), making this assay highly sensitive. However, positive results with a single PCR assay targeting IS481 could lead to a false diagnosis of pertussis because IS481 is also found in *B. holmesii* (8 to 10 copies per genome) (21), in animal isolates of *B. bronchiseptica* (20), and less frequently, in human isolates of *B. bronchiseptica* (17). Moreover, pseudo-outbreaks due to false-positive results of assays using IS481 as a single PCR target have demonstrated the need for defined cutoff values based on analytical sensitivity and clinical relevance (3, 7, 15, 17).

Some data suggest that an increase in respiratory illness caused by *B. parapertussis* is occurring (28). *B. parapertussis* causes a less severe infection than *B. pertussis*. Moreover, pertussis-like illness due to *B. holmesii* occurs in the United States and Canada (10, 16, 29, 32). Infections from *B. holmesii* were recently reported in England and France (7a). In addition, *B. bronchiseptica* is occasionally isolated from the upper respiratory tract of immunocompromised individuals (30). Therefore,

species identification and confirmation of *Bordetella* spp. must occur to allow an accurate diagnosis of pertussis and pertussis-like diseases in humans (9).

The diagnostic needs of clinical versus public health settings are different. In a clinical setting, sensitivity must be optimized while providing rapid results to ensure appropriate treatment and prevention of further transmission. In a public health setting, a high degree of specificity is needed to avoid unnecessary interventions. We previously developed a two-target assay that allowed identification of *B. pertussis*; however, it did not allow confirmatory identification of *B. holmesii*, nor was it as specific or sensitive for *B. parapertussis* (25). The aim of this study was to develop a multitarget RT-PCR assay, using IS481, *B. parapertussis* IS1001 (pIS1001), and *B. holmesii* IS1001-like (hIS1001) targets, that is sensitive and specific and that would allow the identification of *Bordetella* species in a multiplex format and confirmation of these *Bordetella* in a pertussis toxin subunit S1 (*ptxS1*) singleplex format. Although the multitarget assay was not designed for species identification of *B. bronchiseptica*, a presumptive identification using the *ptxS1* target may occur. Thus, the novel multiplex assay in conjunction with the modified *ptxS1* assay would form the multitarget method.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains were obtained from the Centers for Disease Control and Prevention (CDC) culture collections in the Meningitis and Vaccine Preventable Diseases Branch and Respiratory Diseases Branch and from other collaborators at CDC. *B. bronchiseptica* animal isolates were obtained from Karen Register at the United States Department of Agriculture. Four hundred two *Bordetella* species isolates, which included 141 *B. pertussis* isolates, 93 *B. parapertussis* isolates, 78 *B. holmesii* isolates, 73 *B. bronchiseptica* (human host) isolates, 13 *B. bronchiseptica* (animal host) isolates, and 1 isolate each of *B. avium*, *B. hinzii*, *B. petrii*, and *B. trematum*, were grown for 1 to 4 days at 37°C under high humidity on modified Regan-Lowe medium containing charcoal, agar

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TABLE 1. Sequences and optimal concentrations of primers and probes used in the real-time PCR assays

Target	Primer or probe	Sequence (5' → 3')	Amplicon length (bp)	Optimal concn (nM)
IS481 ^a	852U18	CAAGGCCGAACGCTTCAT	66	100
	894L24	GAGTTCTGGTAGGTGTGAGCGTAA		100
	871U22P ^b	CAGTCGGCCTTGCGTGAGTGGG		300
hIS1001 ^c	BHIS41U20	GGCGACAGCGAGACAGAATC	67	100
	BHIS91L17	GCCGCCTTGGCTCACTT		100
	BHIS62U28P ^d	CGTGCAGATAGGCTTTTAGCTTGAGCGC		100
pIS1001 ^e	135U17	TCGAACGCGTGGAATGG	65	300
	199L20	GGCCGTTGGCTTCAAATAGA		300
	157U21P ^f	AGACCCAGGGCGCACGCTGTC		100
ptxS1 ^g	402U16	CGCCAGCTCGTACTTC	55	700
	442L15	GATACGGCCGGCATT		700
	419U22P ⁱ	AATACGTCGACACTTATGGCGA		300
maseP ^h	rnasePforward	CCAAGTGTGAGGGCTGAAAAG	80	400
	rnasePreverse	TGTTGTGGCTGATGAACTATAAAAG		400
	rnasePprobe ^b	CCCAGTCTCTGTGACACTCCCTTC		100

^a GenBank accession no. M28220.

^b Probe 5' end labeled with 6-carboxyfluorescein (FAM) and 3' end labeled with Black Hole Quencher 1 (BHQ1).

^c GenBank accession no. AY786982.

^d Probe 5' end labeled with Quasar 670 and 3' end labeled with Black Hole Quencher 3 (BHQ3).

^e GenBank accession no. X66858.

^f Probe 5' end labeled with hexachlorofluorescein (HEX) and 3' end labeled with Black Hole Quencher 1.

^g GenBank accession no. M14378.

^h GenBank accession no. NM_006413.

ⁱ Probe 5' end labeled with 6-carboxyfluorescein (FAM) and Black Hole Quencher 1 is at position 14 T (underlined). Previously (38), the probe was 3' end labeled with Black Hole Quencher 1.

(Remel, Lenexa, KS), and 10% defibrinated horse blood. Sixty-six non-*Bordetella* species isolates were cultured following standard procedures.

Clinical and spiked specimens. A total of 197 human clinical specimens consisting of nasopharyngeal (NP) aspirates or swabs were submitted to CDC during various cough-illness outbreaks (3–5). Of these specimens, 87 were submitted for culture of *Bordetella* spp. upon arrival (12). Specimens ($n = 17$) spiked with the three most common *Bordetella* spp. from two independent proficiency panels prepared by the ZeptoMatrix Corporation (Buffalo, NY) and the Institut für Medizinische Mikrobiologie und Hygiene (Regensburg, Germany) were also included. After review, this project was considered exempt from institutional review board approval.

DNA methods. DNA extraction from isolates was performed by suspending a swab of the culture in 1 ml of 10 mM Tris-HCl buffer (pH 8.0)–0.85% NaCl solution and boiling for 10 min at 99°C, followed with centrifugation at 20,000 × g for 8 min. The supernatant was collected, and DNA extracts were stored at –20°C until the RT-PCR run. For analytical sensitivity assays, DNA extraction from isolates was performed using a QIAamp DNA minikit (Qiagen, Valencia, CA) following the manufacturer's instructions, and the DNA was eluted in 100 μ l of 10 mM Tris-HCl buffer (pH 8.0). Concentrations of DNA extracted from isolates were determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

DNA extraction from 197 clinical specimens and 17 spiked specimens was performed on MagNA Pure LightCycler equipment using a MagNA Pure LightCycler DNA isolation kit III or total nucleic acid kit (Roche Applied Science, Indianapolis, IN). DNA was extracted from specimens either upon arrival or specimens were stored at –80°C until DNA extraction and testing in the multi-target assay. Human DNA was purchased from Applied Biosystems Inc., Foster City, CA.

Primers and probes. The primers and probes were designed using Primer Express software: version 2.0 for IS481 (25) and version 3.0 for hIS1001 and pIS1001 (Applied Biosystems). The primers and probes were analyzed using the Oligo program (version 6.0; Molecular Biology Insights Inc., Cascade, CO) and synthesized in the Biotechnology Core Facility at CDC (Table 1). The IS481 assay targets a region downstream from the inverted repeat, generating a 66-bp amplicon. In the multiplex assay, 100 nM each primer and 300 nM probe were used, while in the singleplex assay, 300 nM each primer and 300 nM probe were used. The pIS1001 for *B. parapertussis* targets a region at the 5' end of insertion

sequence IS1001 from nucleotides 135 to 199 (GenBank accession no. X66858). The hIS1001 for *B. holmesii* targets a region at the 5' end of the IS1001-like sequence from nucleotides 41 to 107 (GenBank accession no. AY786982).

The ptxS1 primers were described previously (25), and the modified probe is indicated in Table 1.

The maseP primers and probe currently used are listed in Table 1 with their optimal concentrations.

To test the cross-reactivity of *B. bronchiseptica* isolates, commercially available analyte-specific reagents (ASRs) from Cepheid (Sunnyvale, CA) and Roche Applied Science containing primers/probes that target the insertion sequences IS481 and IS1001 (for *B. pertussis* and *B. parapertussis*, respectively) were obtained.

RT-PCR assays. RT-PCR was performed in a total volume of 25 μ l in an optical 96-well plate with an ABI 7500 apparatus (Applied Biosystems). The multiplex amplification mixture contained 4 μ l of extracted DNA plus a 21- μ l reaction mix, using TaqMan Gene Expression PCR master mix (Applied Biosystems), with concentrations of primers and probe optimized for IS481, pIS1001, and hIS1001 (Table 1). The PCR protocol used was as follows: hold for 2 min at 50°C, enzyme activation for 10 min at 95°C, amplification for 15 s at 95°C and 1 min at 60°C, and repeat for 45 cycles. Positive-control samples (containing DNA from *B. pertussis* A639, *B. holmesii* C690, and *B. parapertussis* F585 strains) and non-template-control samples (PCR-grade water) were tested in each run.

The ptxS1 RT-PCR assay was previously described, and an annealing temperature of 57°C (25) and *B. pertussis* strain A639 as a positive control were used. This assay was designed for detection of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*.

The maseP RT-PCR assay was performed using the same amplification conditions used for the multiplex assay and human DNA as a positive control.

The background fluorescence was considered to obtain the correct cycle threshold (C_T) value. Thus, the threshold was drawn above the background fluorescence for each run in the exponential phase of the amplification curve, as recommended by the manufacturer. For the clinical specimens, the threshold settings generally range from 0.02 to 0.2 for all the assays.

Analytical sensitivity assays. The sensitivity of the multiplex RT-PCR assay was measured using serially diluted *B. pertussis*, *B. parapertussis*, and *B. holmesii* isolates. A stock concentration of 20 ng/ μ l of DNA from *B. pertussis* strain A639, *B. parapertussis* F585, and *B. holmesii* C690 was determined on the basis of the

TABLE 2. Algorithm of the multitarget real-time PCR assay

Species	Assay result			
	Singleplex <i>ptxS1</i>	Multiplex		
		IS481	hIS1001	pIS1001
<i>B. pertussis</i>	+	+	-	-
<i>B. parapertussis</i> ^a	+	-	-	+
<i>B. holmesii</i>	-	+	+	-
<i>B. pertussis</i> and <i>B. parapertussis</i> ^a	+	+	-	+
<i>B. pertussis</i> and <i>B. holmesii</i> ^b	+	+	+	-

^a A specimen positive for pIS1001 may be considered to most probably contain *B. parapertussis*, but the possibility that it is positive for *B. bronchiseptica* cannot be totally excluded.

^b This coinfection has been documented by culture in our laboratory.

absorption at 260 nm, and 10-fold serial dilutions were tested in triplicate in the RT-PCR assays to determine the linear dynamic range and the lower limit of detection (LLOD) per PCR based on genomic equivalents for the ABI 7500 assay. Each target DNA with the three sets of primers and probes was compared to each target DNA with one set of specific primers and probe in the same 96-well plate. Experiments were run on three separate days. Within days, two plates were run at three separate time points. Each plate contained two or three replicates. A mixed-model analysis of variance was used to derive means and confidence intervals. The replicate nature of the data was accounted for in the models, which treated day, time point, and plate as random effects; dilution was treated as a fixed effect.

A positive control for the multiplex assay containing DNA from all three strains (A639, C690, and F585) with the three sets of primers and probes was compared to each target DNA with one set of specific primers and probe in the same 96-well plate. The experiments were performed the same as for the multiplex sensitivity experiment to determine the linear dynamic range.

The LLOD of the *ptxS1* assay was performed as for the individual target assays using DNA from *B. pertussis* strain A639.

A model of nonlinear regression (Sigma Plot program, version 9.0) was used for the dynamic range analysis, and the regression line represents data in the linear range. The PCR efficiency (*E*) of the primer pair and probe was calculated using the equation $10^{-1/\text{slope}} - 1$ (27). An *E* of 1.0 indicates that the amplicon quantity is duplicated every cycle.

Analytical specificity assays. The specificity of the multitarget real-time PCR assay was assessed by testing DNA extracts from a collection of non-*Bordetella* spp. (*n* = 66) for cross-reactivity with each individual RT-PCR target, including the *ptxS1* assay, and in the multiplex assay. This collection included *Aerococcus viridans*, *Bacillus cereus*, *B. subtilis*, *Chlamydomypha pneumoniae*, *Corynebacterium diphtheriae*, *C. ulcerans*, *C. accolens*, *C. jeikeium*, *C. minutissimum*, *C. pseudodiphtheriticum*, *C. pseudotuberculosis*, *C. striatum*, *Enterococcus faecalis*, *Escherichia coli*, *Flavobacterium meningosepticum*, *Gemella haemolysans*, *Haemophilus influenzae* serotypes a, b, c, d, e, f, and nontypeable (NT), *H. haemolyticus*, *H. aegyptius*, *H. parainfluenzae*, *Legionella pneumophila*, *L. longbeachae* serogroups 1 and 2, *Moraxella catarrhalis*, *Mycoplasma pneumoniae*, *Neisseria meningitidis* serogroups A, B, C, W135, X, Y, Z, 29E, and NT, *N. sicca*, *N. lactamica*, *N. subflava*, *N. cinerea*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *S. agalactiae*, *S. canis*, *S. anginosus*, *S. equi*, *S. zooepidemicus*, *S. porcinus*, *S. constellatus*, *S. iniae*, *S. intermedius*, *S. pseudopneumoniae*, *S. mitis*, *S. oralis*, *S. sanguinis*, *S. salivarius*, *S. pyogenes*, *S. agalactiae*, *S. bovis*, and *S. dysgalactiae*. In addition, cross-reactivity with serial dilutions of human genomic DNA (10 ng/μl; Applied Biosystems) was tested for all RT-PCR assays.

DNA from 402 *Bordetella* species was used in the individual evaluation of each RT-PCR target assay and in the multiplex assay for cross-reactivity at a 5-ng/μl concentration.

RT-PCR of clinical and spiked specimens. One hundred ninety-seven clinical and 17 spiked specimens were tested with the multiplex and the *ptxS1* assays in duplicate and with one 1-to-5 dilution of the specimen. Eighty-seven clinical and all spiked specimens were evaluated with both the singleplex and multiplex assays on the same 96-well reaction plate according to the algorithm in Table 2. The *ptxS1* assay was performed in a separate reaction for all specimens. A water control was placed between every sample. An average *C_T* value of the duplicate RT-PCR assays was calculated to give a final value. If a specimen was positive in two of three tests, it was considered positive. If a specimen was positive with a *C_T* value of ≥ 35 in only one of three tests for IS481, it was considered negative.

Clinical specimens were also tested for the human *maseP* gene using the RT-PCR assay to monitor the quality of DNA in the specimen and to check for inhibition. To be considered positive for *maseP*, a specimen had to have a *C_T* value of <40 . If *maseP* is negative, a single 1-to-5 dilution of the specimen in water was tested by the *maseP* assay.

DNA sequencing. PCR primers IS481-5 (5'-GATTCAATAGGTTGTATGC ATGGTT-3') and IS481-12 (5'-TCGTCCAGGTTGAGTCTGGA-3') were used to amplify a 1,014-bp fragment of IS481 by conventional PCR (20). PCR primers IS1001-135F (5'-TCGAACGCGTGAATGG-3') and IS1001-1157R (5' CCA GGATGCCGTGCAGAT-3') were used to amplify a 1,023-bp fragment of IS1001 from the pIS1001 RT-PCR-positive *B. bronchiseptica* strains. The Expand High Fidelity PCR system (Roche Applied Science) was utilized with 300 nM each primer. The DNA was denatured at 95°C for 5 min and subjected to 40 cycles of amplification (95°C for 30 s, 55°C for 30 s, 72°C for 45 s), followed by a final extension of 7 min at 72°C. The PCR products were purified with a QIAquick PCR purification kit (Qiagen, Valencia, CA).

Fluorescence-based cycle sequencing with a BigDye Terminator cycle sequencing kit (version 3.1; Applied Biosystems) was performed for both DNA strands in the presence of 1.0 M betaine and 5% dimethyl sulfoxide. The products of the sequencing reactions were purified on a Centri-sep 96-well gel filtration plate (Princeton Separations, Adelphia, NJ) and were separated on an AB3130xl system (Applied Biosystems). The data were analyzed with the GCG or Lasergene (version 8) (DNASTar) software package.

Southern analysis. Two micrograms of purified genomic DNA from *B. pertussis* A639, *B. parapertussis* F585, *B. bronchiseptica* BBE001, D982, and F579 (human isolates), and *B. bronchiseptica* MBORD731, MBORD668, and RB50 (animal isolates) was digested with PstI and NarI (New England BioLabs, Inc., Ipswich, MA). Each set of DNA digests was separated on a 0.8% agarose gel and transferred to a nylon membrane. One kilobase digoxigenin-labeled probes was prepared from PCR-amplified products of the IS481 region from *B. pertussis* A639 IS481 variant 1 and *B. bronchiseptica* BBE001 IS481 variant 2 with a digoxigenin DNA labeling kit (Roche Applied Science). Hybridization was performed under stringent conditions with each probe, and DNA bands were visualized using the digoxigenin DNA detection kit (Roche Applied Science).

RESULTS

***Bordetella* species differentiation by multitarget RT-PCR assay.** The multitarget RT-PCR assay allowed discrimination between *Bordetella* species isolates, as predicted by the algorithm (Table 2). All 141 *B. pertussis* isolates were positive for IS481 and *ptxS1* and negative for pIS1001 and hIS1001. All 93 *B. parapertussis* isolates were positive for *ptxS1* and pIS1001 and negative for hIS1001 and IS481. All 78 *B. holmesii* isolates were positive for IS481 and hIS1001 and negative for pIS1001 and *ptxS1*. *B. avium*, *B. hinzii*, *B. petrii*, and *B. trematum* isolates were negative for all targets.

Of the 73 human-derived *B. bronchiseptica* strains, 72 were negative for IS481, but 1 human isolate, *B. bronchiseptica* strain BBE001, was positive for IS481 with a high *C_T* value (38.5), positive for *ptxS1* with a low *C_T* value (27.0), and negative for pIS1001. DNA sequencing demonstrated that three differences between the IS481 reverse primer and the sequence in the strain explaining the high *C_T* value were found (Fig. 1). Sequencing of the 1-kb IS481 fragment from the *B. bronchiseptica* human isolate demonstrated the presence of IS481 variant 2 that is found in *B. bronchiseptica* animal isolates (20) and is available in the GenBank database (GenBank accession no. HQ692885; <http://www.ncbi.nlm.nih.gov/GenBank/index.html>). Southern blotting confirmed the presence of IS481 in the *B. bronchiseptica* human isolate (data not shown). Forty-seven of the *B. bronchiseptica* isolates were *ptxS1* positive, and 26 were negative.

Of the 13 animal-derived *B. bronchiseptica* strains, 6 were IS481 negative and 7 were IS481 positive. Among the seven IS481-positive strains, one had a low *C_T* value (IS481 variant 1)

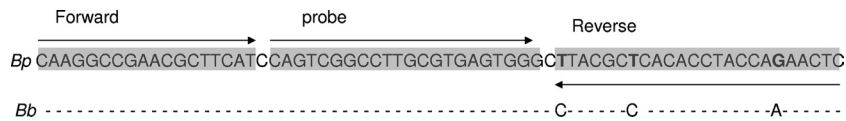


FIG. 1. Sixty-six nucleotides of DNA sequence from IS481 region of *B. pertussis* targeted with the IS481 RT-PCR primers used in this study. Three nucleotide changes are observed in the IS481 region from *B. bronchiseptica* (Bb) (BbIS481variant2) compared to IS481 from *B. pertussis* (Bp) (BpIS481variant1) where the *B. pertussis* IS481 real-time reverse primer anneals. Bp, IS481 region of *B. pertussis*; Bb, IS481 region from *B. bronchiseptica* human isolate BBE001 with the three nucleotide differences indicated. Arrows, locations where the *B. pertussis* IS481 forward and reverse primers and probe from the IS481 RT-PCR assay anneal.

and six had high C_T values (IS481 variant 2) (20). Twelve were *ptxS1* positive. Five *B. bronchiseptica* strains (four human derived and one animal derived) were negative by IS481 but cross-reacted with pIS1001. The strains were also positive for *ptxS1* and could be misidentified as *B. parapertussis* on the basis of the algorithm. These strains were confirmed to be *B. bronchiseptica* by biochemical tests. The same five *B. bronchiseptica* isolates and one additional *B. bronchiseptica* human isolate reacted with the IS1001 primers/probe from the two commercial sources. These commercial kits detect both *B. pertussis* and *B. parapertussis* with IS481 and IS1001 primer/probe sets, respectively. Partial DNA sequencing of IS1001 regions from two of five *B. bronchiseptica* strains that cross-reacted in the pIS1001 assay demonstrated that the strains contained the same nucleotide sequence found in *B. parapertussis* (data not shown).

All 86 *B. bronchiseptica* isolates were negative with the hIS1001 primers/probe.

Analytical specificity of RT-PCR assays. All 66 isolates of non-*Bordetella* species and human DNA were negative by use of the IS481, pIS1001, hIS1001, and *ptxS1* targets.

Analytical sensitivity of RT-PCR assays. The IS481 assay achieved >99% efficiency with linear amplification over a 6-log-unit dynamic range for *B. pertussis* in the singleplex and multiplex formats (Fig. 2A) and over a 5-log-unit dynamic range for *B. holmesii* in the singleplex and multiplex formats (data not shown). The pIS1001 and the hIS1001 assays achieved >99% efficiency with linear amplification over a 5-log-unit dynamic range for *B. parapertussis* (Fig. 2B) and for *B. holmesii*, respectively (Fig. 2C), in the singleplex and multiplex formats. For the IS481, pIS1001, and hIS1001 assays, the regression coefficients ($R^2 = 0.99, 0.99, \text{ and } 0.99$, respectively) and the amplification efficiencies ($E = 1.08, 1.27, \text{ and } 1.27$, respectively) demonstrated an exponential amplification of DNA with the primer and probe sets (Fig. 2A to C) for the multiplex assay. For the IS481, pIS1001, and hIS1001 assays, the regression coefficients ($R^2 = 0.99, 0.988, \text{ and } 1.00$, respectively) and the amplification efficiencies ($E = 1.06, 1.24, \text{ and } 1.03$, respectively) demonstrated an exponential amplification of DNA with the primer and probe sets (Fig. 2A to C) for the singleplex assays.

The LLOD for IS481, pIS1001, and hIS1001 targets is <1 genomic equivalent per RT-PCR for the singleplex and multiplex assays (Table 3).

The combination of the three DNA targets with three primer/probe sets achieved efficiencies in the range from 81% to 99% over a 7-log-unit dynamic range, demonstrating the utility of this assay as a positive control for the multiplex assay (data not shown).

The LLOD for the *ptxS1* target is <10 genomic equivalents per RT-PCR for the singleplex assay (Table 3). The *ptxS1* assay achieved >99% efficiency with linear amplification over a 5-log-unit dynamic range for *B. pertussis* in the singleplex assay (data not shown). The regression coefficient ($R^2 = 0.99$) and the amplification efficiency ($E = 1.03$) for the *ptxS1* assay demonstrated an exponential amplification of DNA with the primer and probe set (data not shown).

Detection of *Bordetella* spp. in blinded study of spiked specimens. Of the 17 samples, 8 contained *B. pertussis* with concentrations ranging from 1 to 10,000 genomic equivalents, 2 were *B. holmesii* with C_T values of <35 for both targets, 2 were *B. parapertussis*, 1 was a mixture of *B. pertussis* and *B. parapertussis*, and 4 were negative for *Bordetella* spp. The C_T values in the multiplex assay for the samples were within 1 C_T value of those found in the singleplex assays. Every specimen was correctly identified with the multitarget assay.

Detection of *Bordetella* spp. in NP specimens by culture and RT-PCR. In the blinded retrospective study, 87 NP specimens collected during 6 cough-illness outbreaks were tested using the multitarget assay. A total of 19.6% of the 80 NP specimens were positive for *Bordetella* spp. by culture, whereas 24% of the 87 NP specimens were positive for *Bordetella* spp. by RT-PCR. Moreover, all 25 specimens that generated C_T values gave comparable results in both the singleplex and the multiplex assays, supporting the accuracy of the multiplex assay. Of the 25 clinical specimens that generated C_T values, 4 specimens had 2 of 3 replicates with a high C_T value (C_T values, ≥ 35) with IS481 alone and were interpreted to have an indeterminate result. One sample with an indeterminate result by PCR was culture positive, while four PCR-positive NP specimens were culture negative, demonstrating the greater sensitivity of PCR. Sixty-two (71.3%) clinical specimens were RT-PCR and culture negative.

Fifteen (17%) of the specimens showed amplification of IS481 but no amplification of either hIS1001 or pIS1001. The specimens were confirmed to be *B. pertussis* by the *ptxS1* assay. Ten of the specimens were positive by culture, three were negative, and two specimens were unavailable for cultivation.

Two clinical specimens were culture positive for *B. parapertussis* and RT-PCR positive for both *B. pertussis* by the IS481 assay (C_T values, <35) and *B. parapertussis* by the pIS1001 assay. The specimens were also *ptxS1* positive, suggesting the presence of both *B. pertussis* and *B. parapertussis*. Two samples were positive for *B. holmesii* by the hIS1001 and IS481 assays in the multiplex RT-PCR (C_T values, <35) and negative by the *ptxS1* assay, which is interpreted to be an infection by *B. holmesii*.

Two specimens were negative in the multiplex assay but

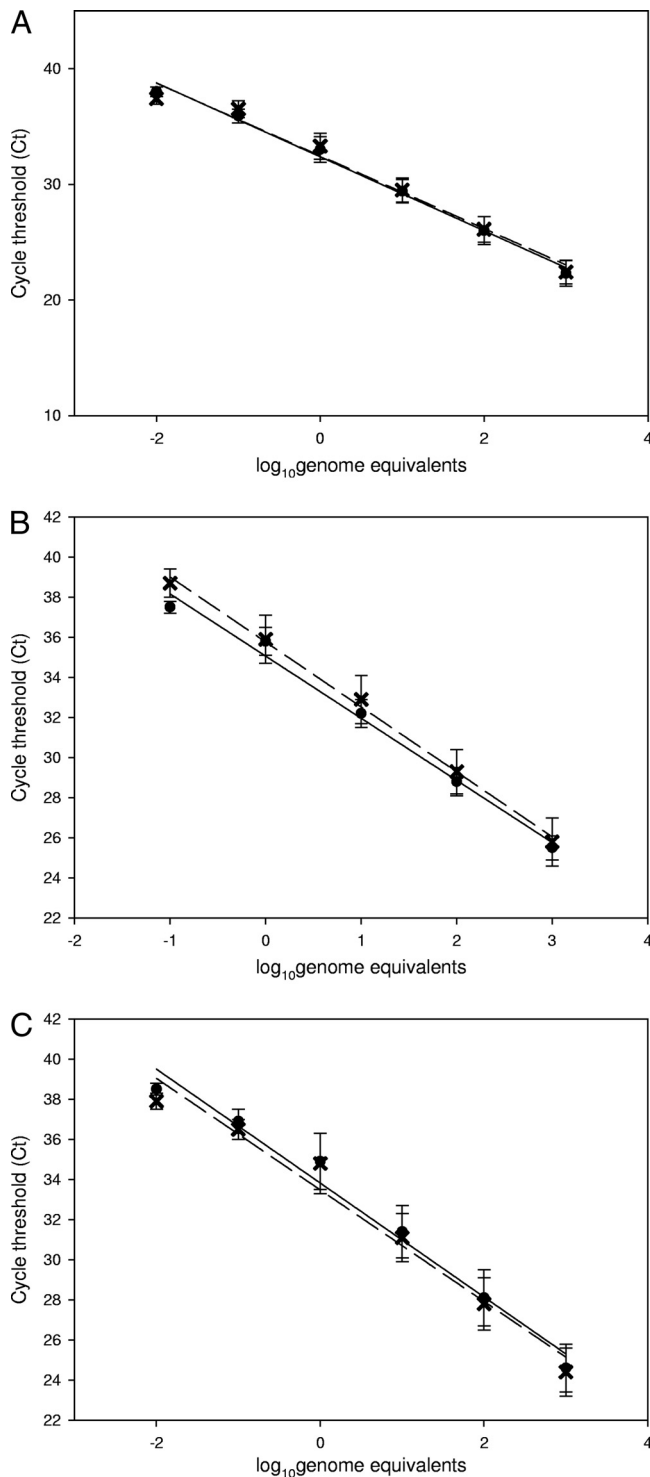


FIG. 2. Linear dynamic range of the singleplex and multiplex RT-PCR assays using *Bordetella* DNAs. (A) IS481 primers/probe with *B. pertussis* A639 DNA. ●, singleplex assay; the threshold settings for the IS481 primer/probe set range from 0.05 to 0.15; IS481/hIS1001/pIS1001 primers/probes with *B. pertussis* A639 DNA; ×, multiplex assay; the threshold settings for the IS481 primer/probe set range from 0.07 to 0.11. (B) pIS1001 primers/probe with *B. paraperussis* F585 DNA. ●, singleplex assay; the threshold settings for the pIS1001 primer/probe set range from 0.04 to 0.07; IS481/hIS1001/pIS1001 primers/probes with *B. paraperussis* F585 DNA; ×, multiplex assay; the threshold settings for the pIS1001 primer/probe set range from 0.04 to 0.05.

positive by the *ptxS1* assay. The results for these specimens were suggestive of *B. bronchiseptica*, although no confirmatory tests were performed due to an insufficient quantity of the specimens. The *maseP* results, with a range of C_T values from 21.7 to 36.4, demonstrated amplifiable human DNA in all specimens.

Detection of *Bordetella* spp. in NP specimens by RT-PCR. Of the remaining 110 clinical specimens from pertussis-like outbreaks occurring from 2008 to 2011 tested at CDC with the multitarget RT-PCR assay, 89 NP specimens were from the United States and 21 were from other countries. A total of 36 (32.7%) of the 110 NP specimens were positive for *Bordetella* spp. by the RT-PCR assay.

Of the 24 specimens positive for *B. pertussis*, 4 were *ptxS1* assay negative but had IS481 assay C_T values from 32 to 34 and were considered positive (Table 4). Seven specimens were positive for *B. holmesii* and one was positive for a coinfection of *B. pertussis* and *B. holmesii*, with C_T values of less than 35 for the IS481, hIS1001, and *ptxS1* assays. Four specimens were positive by both the pIS1001 and *ptxS1* assays and were most probably *B. paraperussis*, but the possibility that they were *B. bronchiseptica* cannot be excluded. Five specimens were considered indeterminate, with IS481 assay C_T values of greater than or equal to 35, and 69 were negative for *Bordetella* spp.

RT-PCR interpretation. The interpretation criteria for RT-PCR results during outbreaks of cough illness based on the results of the multitarget assays are stated in Table 4. The RT-PCR assay result was considered negative if the C_T value was greater than or equal to 40. A specimen was considered positive for *B. pertussis* DNA by RT-PCR if it was positive (any C_T value of less than 40) for the IS481 and *ptxS1* targets and negative for both the pIS1001 and hIS1001 targets. If a specimen was *ptxS1*, hIS1001, and pIS1001 target negative with an IS481 assay C_T value of less than 35, it was considered *B. pertussis*. If a specimen was *ptxS1*, hIS1001, and pIS1001 target negative with an IS481 assay C_T value of greater than or equal to 35 but less than 40, it was considered indeterminate. If a specimen was positive for both the IS481 and hIS1001 targets and *ptxS1* assay negative, it was considered *B. holmesii*; however, the possibility that a very low level of *B. pertussis* is present cannot be totally excluded. If a specimen was negative for IS481 and positive for pIS1001, it was most probably positive for *B. paraperussis* DNA, but the possibility that it is *B. bronchiseptica* cannot be totally excluded. If a specimen was positive for *ptxS1* but negative for IS481, pIS1001, and hIS1001, then it was suggestive of *B. bronchiseptica*.

DISCUSSION

This report documents how a multitarget approach incorporating a multiplex and singleplex assay can be used to increase

(C) hIS1001 primers/probe with *B. holmesii* C690 DNA. ●, singleplex assay; the threshold settings for the hIS1001 primer/probe set range from 0.06 to 0.09, IS481/hIS1001/pIS1001 primers/probes with *B. holmesii* C690 DNA; ×, multiplex assay; the threshold settings for the hIS1001 primer/probe set range from 0.03 to 0.05. The regression lines represent data in the linear range. Error bars equal the 95% confidence interval.

TABLE 3. LLODs of individual and multiplex real-time PCR assays

Assay and genomic equivalent	Mean C_T (95% CI ^a)				
	<i>B. pertussis</i> IS481	<i>B. holmesii</i> IS481	<i>B. holmesii</i> IS1001-like	<i>B. parapertussis</i> IS1001	<i>ptxS1</i> ^b
Individual RT-PCRs					
1,000	22.3 (18.0–26.9)	24.6 (18.9–30.2)	24.6 (19.3–29.9)	25.5 (22.8–28.1)	30.1 (29.9–30.2)
100	26.0 (21.1–31.0)	28.1 (21.9–34.3)	28.1 (22.0–34.1)	28.8 (25.8–31.8)	33.4 (33.1–33.6)
10	29.4 (24.9–33.9)	31.7 (25.5–37.9)	31.4 (25.7–37.0)	32.2 (29.3–35.2)	37.1 (36.9–37.4)
1	33.0 (28.2–37.9)	34.8 (30.3–39.3)	34.9 (28.8–41.0)	35.8 (32.6–39.1)	38.9 (38.5–39.4) ^c
0.1	35.9 (33.4–38.4)	36.7 (34.8–38.6)	37.0 (34.4–39.5)	37.5 (35.5–39.5)	ND ^d
0.01	38.0 (35.8–40.3)	ND	38.5 (37.4–39.6)	ND	ND
0.001	ND	ND	ND	ND	ND
Multiplex RT-PCR					
1,000	22.4 (18.0–26.9)	25.1 (19.0–31.1)	24.4 (19.1–29.6)	25.8 (20.8–30.8)	
100	26.1 (21.3–30.9)	28.6 (22.3–35.0)	27.8 (22.0–33.6)	29.3 (24.4–34.2)	
10	29.5 (25.0–34.0)	32.1 (26.3–38.0)	31.1 (25.9–36.3)	32.9 (27.8–38.0)	
1	33.3 (28.5–38.1)	35.8 (30.4–41.1)	34.8 (28.3–41.2)	35.9 (30.8–40.9)	
0.1	36.5 (33.3–39.7)	37.6 (35.3–40.0)	36.5 (33.6–39.4)	38.7 (35.6–41.8)	
0.01	37.4 (36.1–38.8)	ND	37.9 (32.3–43.6)	ND	
0.001	ND	ND	ND	ND	

^a CI, confidence interval.

^b *ptxS1* probe has Black Hole Quencher 1 on the position 14 T (underlined) (Table 1).

^c Seventeen of 32 tests gave C_T values generating this mean.

^d ND, not detected.

the specificity of PCR testing for *B. pertussis* diagnosis by distinguishing between three *Bordetella* species: *B. pertussis*, *B. parapertussis*, and *B. holmesii*. A few assays using more than one target per reaction have been published (1, 6, 10, 22, 23, 26). The region of IS481, pIS1001, and hIS1001 targeted in our assay is sensitive, with LLODs of ≤ 1 *Bordetella* genomic equivalent per reaction (Fig. 2A to C), and is also specific, as no cross-reactivity with non-*Bordetella* species or human DNA occurs.

Interpretation of the single-target IS481 RT-PCR assay result is problematic when high C_T values are obtained. Since IS481 is a multicopy insertion sequence, it is a potentially advantageous target because it may be more sensitive; however, this feature also may make it more prone to generate false-positive results. False-positive results are more likely with high-copy-number targets because contaminating DNA is more readily amplified to give a positive result. These results can lead to a misdiagnosis, particularly during outbreak investigations, when large numbers of samples are tested in a short period of time (3, 7, 15, 17).

In our study, we found 9 (4.6%) clinical specimens that had high-positive IS481 assay C_T values ($35 \leq C_T < 40$) but were

negative for the hIS1001, pIS1001, and *ptxS1* targets. The RT-PCR results of these specimens were considered indeterminate (Table 4). On the basis of our analytical sensitivity data in Table 3, where the average C_T value for 1 genomic equivalent is 33.0, IS481 C_T values in the range of $35 \leq C_T < 40$ indicate the presence of less than 1 bacterium per reaction, which we consider uninterpretable. For the single-copy target of *ptxS1*, the average C_T value for 1 genomic equivalent is 38.9 (Table 3). Thus, for single-copy targets, including the *maseP* target, a C_T cutoff of 40 was implemented. In outbreak settings, RT-PCR results must be correlated with the results of other laboratory tests, such as culture and serology, or with clinical and epidemiological data before the outbreak is considered confirmed to have been caused by *B. pertussis*. This strategy may avoid overdiagnosis of pertussis and the consequent unnecessary implementation of an extensive public health response and control measures (3, 15). For these reasons, we propose using the four-target algorithm for determining the presence of *B. pertussis* by RT-PCR in outbreak situations and inclusion of IS481 cutoff values for result interpretation (11, 18, 24). In a clinical laboratory, sensitivity is a more critical parameter, and the implication of high C_T values may be addressed less strin-

TABLE 4. Interpretation of real-time PCR algorithm during pertussis outbreaks

	Result for:				Interpretation
	IS481	pIS1001 ^a	hIS1001 ^a	<i>ptxS1</i> ^a	
$C_T < 35$		Negative	Negative	Positive or negative	<i>B. pertussis</i>
$35 \leq C_T < 40$		Negative	Negative	Negative	Indeterminate ^b
Positive		Negative	Positive	Negative	<i>B. holmesii</i>
Negative		Positive	Negative	Positive	<i>B. parapertussis</i> ^c
Negative		Negative	Negative	Positive	<i>B. bronchiseptica</i> ^d

^a A C_T of < 40 is considered a positive reaction; a C_T of ≥ 40 is considered negative.

^b Requires confirmation by other means (culture, serology, or epidemiological linkage).

^c A specimen positive for pIS1001 may be considered to most probably contain *B. parapertussis*, but the possibility that it is positive for *B. bronchiseptica* cannot be totally excluded.

^d Of the human-derived *B. bronchiseptica* isolates, 64.4% were positive with *ptxS1*.

gently. In either an outbreak situation or a clinical laboratory, the interpretation of high C_T values is difficult, as these results can be real positives or false positives due to contamination from the environment or laboratory. These challenges reaffirm the need to adopt cutoff values based on the sensitivity of the assay.

As shown in Table 4, use of the combination of four RT-PCR targets in two separate reactions is a useful approach to detect *Bordetella* spp. in clinical specimens collected during respiratory illness outbreaks. For this reason, in addition to the insertion sequences, primers and probe that target the coding region of subunit 1 of the pertussis toxin gene (*ptxA* gene) are maintained in our diagnostic algorithm to enhance species determination, to increase the accuracy of *B. pertussis* DNA detection by RT-PCR (25), and to determine coinfections (Table 2). One potential limitation of the multitarget assay occurs if very low levels of *B. pertussis* are present with *B. holmesii*, which would give the same pattern as that for *B. holmesii* alone: *ptxS1* and *pIS1001* negative and *IS481* and *hIS1001* positive. However, the clinical relevance of this situation with very low levels of *B. pertussis* DNA is unclear.

Coinfections of *B. parapertussis* and *B. pertussis* have been documented (14). In our clinical specimens, two coinfections of *B. pertussis* and *B. parapertussis* were potentially identified, with *IS481* C_T values of less than 35 and *pIS1001*-positive results suggesting that the specimens were most probably coinfections of the two species. These results exemplify the robustness of the multiplex assay. Although we identified one coinfection of *B. holmesii* and *B. pertussis* (*IS481*, *hIS1001*, and *ptxS1* positive and *pIS1001* negative) using our PCR multitarget assay, it was not culture confirmed. However, we recently identified a coinfection of *B. holmesii* and *B. pertussis* by culture (unpublished data). For coinfections of *B. pertussis* and *B. parapertussis* or *B. holmesii*, our RT-PCR data are very limited; however, C_T values for the appropriate targets of less than 40 with a *ptxS1* assay-positive result are suggestive of coinfections.

The results from testing of 402 characterized clinical isolates substantiate the value of our algorithm to discriminate among *Bordetella* species. Since our *pIS1001* primers/probe cross-reacted with 4 human-derived *B. bronchiseptica* isolates (5.5%), all *B. bronchiseptica* isolates may not be distinguished from *B. parapertussis* by this algorithm. In *B. bronchiseptica*, the presence of *IS481* is host dependent (20), and human isolates of *B. bronchiseptica* may contain *IS481* as well (17). Using our primers, we found only 1 of 73 human *B. bronchiseptica* isolates (strain BBE001, isolated in 1956) with *IS481* homology, but the amplification results were atypical and none of the more recent human isolates in our collection had *IS481* detectable by our RT-PCR assay. Genome sequencing of two human-derived *B. bronchiseptica* isolates, BBE001 and BBF579 (isolated in 2007), was performed (13). These results confirmed the *IS481* sequences (Fig. 1) for BBE001 and demonstrated that BBF579 did not contain *IS481* sequences.

Some laboratories use virulence genes in RT-PCR assays to determine the presence of *B. pertussis* DNA. It was recently found (2) that one *B. pertussis* isolate did not express pertussis toxin and four isolates did not express pertactin because the genes for these proteins were deleted. Isolates such as these would confound diagnostics based solely on these virulence genes. Our multiplex assay would alleviate any discrepancy by

incorporating the *IS481* and additional targets for *B. holmesii* and *B. parapertussis*, which would allow the clinical laboratory to more accurately diagnose *B. pertussis* infection and discriminate among other *Bordetella* species. Thus, a multitarget assay including the multicopy insertion sequences for the three *Bordetella* spp. is advantageous.

In summary, a new multitarget approach which includes a multiplex RT-PCR test in combination with a *ptxS1* assay was developed to improve pertussis and pertussis-like illness diagnosis. The multitarget approach as well as the RT-PCR recommendations for cutoff values will be further validated using culture and serologic assays in an ongoing prospective clinical study involving CDC, state and local public health departments, and emerging infections program sites.

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