

## Real-Time PCR Assay Targeting IS481 of *Bordetella pertussis* and Molecular Basis for Detecting *Bordetella holmesii*

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**Detection of *Bordetella holmesii* by a real-time PCR assay targeting IS481 of *Bordetella pertussis* is reported. Sequencing of IS481-specific PCR products from *B. pertussis* and *B. holmesii* isolates revealed sequence homology. Restriction fragment length polymorphism demonstrated a low copy number of IS481-like sequences in *B. holmesii*. These results, and culture of *B. holmesii* from patients with cough, suggest that the specificity and predictive value of IS481-based PCR assays for pertussis may be compromised.**

*Bordetella pertussis* is the causative agent of whooping cough, an infectious disease that occurs worldwide with a high prevalence among young, unvaccinated infants (2, 3, 6, 19) and is recently resurgent in highly vaccinated populations. Related species, including *B. parapertussis*, *B. bronchiseptica*, and the more recently described *B. holmesii* (27), may also cause a pertussis-like syndrome in humans. Laboratory diagnosis of pertussis is traditionally based primarily on culture, which is highly specific but is maximally sensitive only in the initial phases of disease (10, 14). Furthermore, culturing depends on specimen quality and laboratory expertise and requires special media, extended incubation periods of 7 days or more, and confirmation by biochemical or antibody reagent tests. Diagnostic serology can be highly sensitive and more rapid than culture (14), but no serologic assay has been approved for diagnostic use in the United States because no diagnostic criterion has been widely accepted and no method has been validated between laboratories.

Thus, there is a need for more rapid and sensitive diagnostic methods that have high positive predictive value, especially in the early stages of disease. As is the case for other fastidious organisms, PCR offers an attractive alternative for detecting *B. pertussis* and *B. parapertussis* in clinical specimens (1, 4, 5, 7, 15, 17, 21, 26). Previously evaluated *B. pertussis* target regions include insertion sequences (IS), repeat elements, the pertussis toxin promoter region, the adenylate cyclase gene, and the porin gene. IS481 is present in the *B. pertussis* genome at 80 (22) to 100 (5, 18) copies, and PCR assays targeting IS481 have been evaluated for sensitivity and specificity in several laboratories over the last few years. Therefore, an internal region of IS481 was selected as the *B. pertussis* target (18) while a Light-Cycler (Roche Molecular Biochemicals, Mannheim, Germany) duplex PCR for *B. pertussis* and *B. parapertussis* was being developed. The previously described (5) forward primer BP-1 (5' GAT TCA ATA GGT TGT ATG CAT GGT T 3' and the

slightly modified reverse primer BP-2 (5' TTC AGG CAC ACA AAC TTG ATG GGC G 3'), corresponding to bp 12 to 36 and 192 to 167 (GenBank M22031) of IS481, respectively, were used for amplification. A pair of fluorescence-labeled hybridization probes, BP-HP-3 (5' TCG CCA ACC CCC CAG TTC ACT CA-FAM 3') and BP-HP-4 (5' LC red 640-AGC CCG GCC GGA TGA ACA CCC-3'-phosphate), corresponding to bp 66 to 88 and 92 to 112 (GenBank M22031) of IS481, respectively, were used for the real-time detection of IS481-specific PCR products. Amplification mixtures contained 2  $\mu$ l of 10 $\times$  LightCycler FastStart DNA Master Hybridization Probes mix (Roche Molecular Biochemicals), 3 mM MgCl<sub>2</sub>, 0.5  $\mu$ M concentrations of each primer oligonucleotide, 0.2  $\mu$ M concentrations of each hybridization probe oligonucleotide, and 2  $\mu$ l of template DNA in a final volume of 20  $\mu$ l. Following an initial denaturation at 95°C for 10 min, the 50-cycle amplification profile consisted of heating at 20°C/s to 95°C with a 10-s hold, cooling at 20°C/s to 50°C with a 10-s hold, and heating at 20°C/s to 72°C with a 20-s hold.

We evaluated the specificity of this primer-and-probe combination with the following: the type strains *B. pertussis* ATCC 11615 (American Type Culture Collection, Manassas, Va.) and *B. holmesii* ATCC 51541; isolates of *B. pertussis* ( $n = 15$ ), *B. parapertussis* ( $n = 10$ ), *B. bronchiseptica* ( $n = 2$ ), *B. holmesii* ( $n = 5$ ), and *B. trematum* ( $n = 1$ ); and 80 gram-positive and -negative organisms other than *Bordetella* spp. ( $n = 80$ ). A table of all isolates used will be provided upon request. Positive PCR results, as evidenced by probe fluorescence, were observed not only with the 16 *B. pertussis* isolates but also with *B. holmesii* type strain ATCC 51541 and all five *B. holmesii* clinical isolates (Fig. 1). No other isolates tested positive. The decrease in fluorescence intensity observed at cycle numbers above 25 is caused by the "hook effect," when the number of amplicon molecules exceeds the number of fluorescence-labeled hybridization probe molecules present in the reaction mixture. This observation is in agreement with recent studies that detected *B. holmesii* genomic DNA by IS481-based PCR assays (8, 11, 28). We used direct sequencing of the respective 181-bp product amplified from the 1,053-bp IS481 coding sequence with all *B. pertussis* and *B. holmesii* isolates to demonstrate the molecular basis for this result: the two species share

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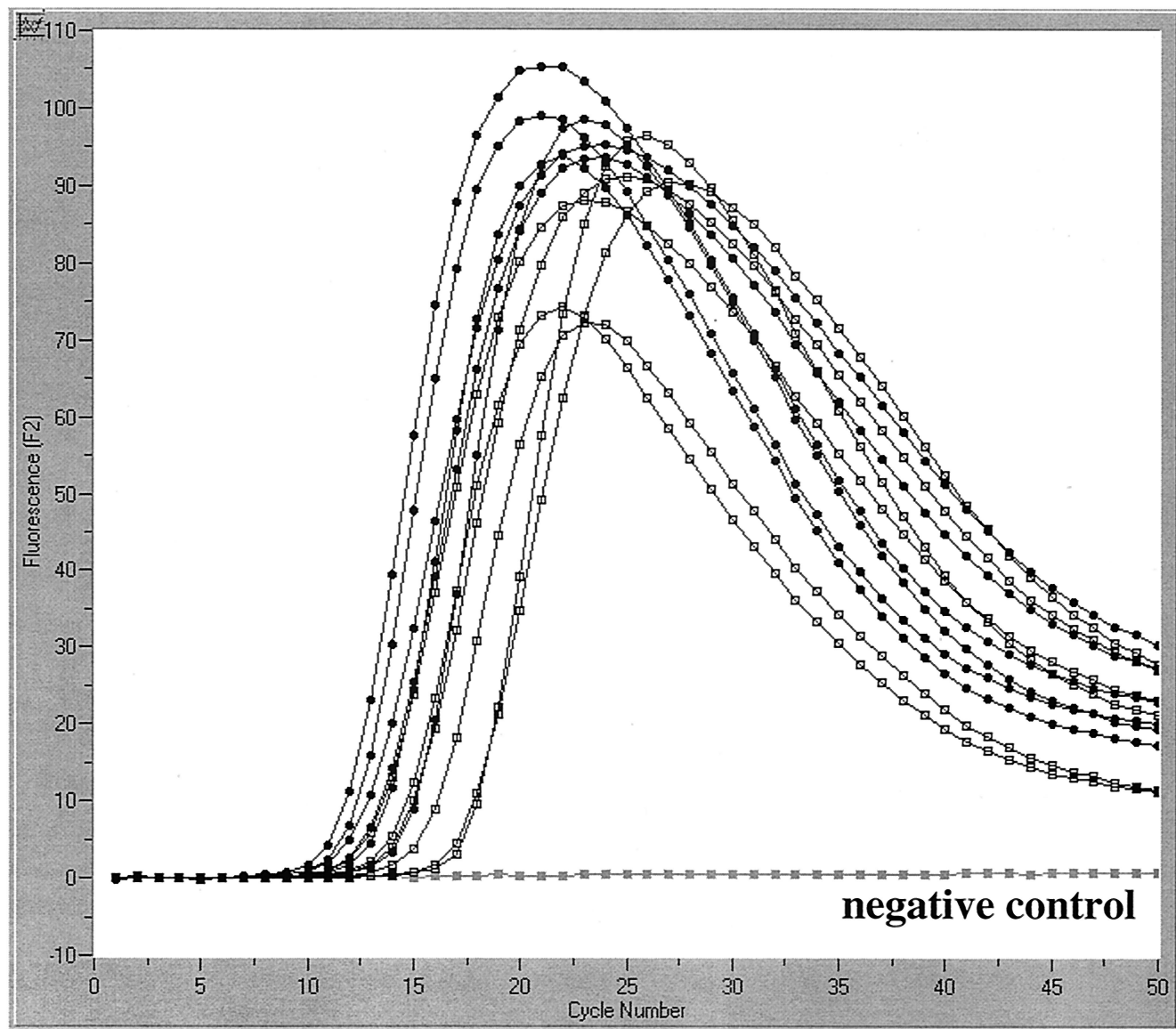


FIG. 1. Evaluation of the *IS481*-specific LightCycler PCR assay with a set of six cultured strains of *B. pertussis* (type strain ATCC 11615 and five different patient isolates) and six cultured strains of *B. holmesii* (type strain ATCC 51541 and five different patient isolates [00, 91, 95, 96, and 98]; kindly provided by H. George, Massachusetts Dept. of Public Health). Amplicon curves represent *B. pertussis* (●) and *B. holmesii* (□) strains.

identical sequences within the amplified region of *IS481*. Sequence identity was further confirmed by sequencing cloned amplicons and 1,010-bp *IS481* amplicons of *B. pertussis* and *B. holmesii* type strains (data not shown). Ambiguities were seen at nucleotide positions 133 (M) and 238 (Y) in all six *B. holmesii* isolates that may result from A/C or C/T variation, respectively, within the different *IS481* alleles in the genome.

To further investigate the number and distribution of *IS481*-like sequences within the respective genomes of *B. pertussis* and *B. holmesii*, we used restriction fragment length polymorphism (RFLP) and Southern blot analysis. Bacterial strains were grown as described previously (24) and DNA extraction, digestion of chromosomal DNA with *Pvu*II, and Southern blotting of restriction fragments were performed according to reported protocols (23, 25). The *IS481* probe was labeled by

direct incorporation of digoxigenin-dUTP during PCR (16) using primers BP-1 and BP-2. Southern blot membranes were exposed to X-ray films, and hybridizations were visualized by chemiluminescence. RFLP analysis demonstrated that *IS481*-specific sequences were present in high copy numbers (>50) in *B. pertussis* but in only 8 to 10 copies in *B. holmesii* (Fig. 2). This suggests that *IS481* has been present in *B. pertussis* for a longer time than in *B. holmesii*. Although *IS481* may have been inherited from an ancestor common to both *B. pertussis* and *B. holmesii*, recent evidence of a shared anatomic site (28) suggests the opportunity for the horizontal transfer of this element (22).

Amplification of targets within insertion sequences is attractive because their high genomic copy number (50 to 100 copies) allows increased assay sensitivity compared to single-copy

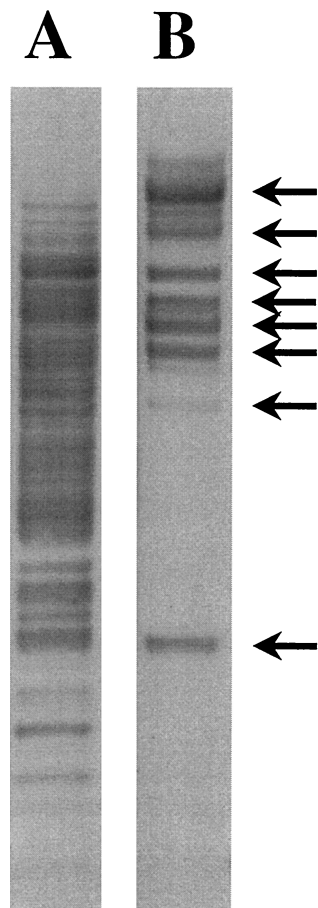


FIG. 2. RFLP and Southern blot analysis for IS481 sequences. Chromosomal DNA of *B. pertussis* ATCC 11615 (A) and *B. holmesii* ATCC 51541 (B) was digested with *Pvu*II, and the blot was hybridized with an IS481-specific probe. *B. holmesii* DNA fragments containing IS481-like sequences are indicated by arrows.

targets. However, the insertion sequences of the eubacteria are transposable DNA elements capable of horizontal transfer across species and spontaneous elimination (29), characteristics that tend to decrease assay specificity and sensitivity, respectively. Due to the presence of IS481-like sequences in *B. holmesii*, a simple modification of PCR assay parameters or targeting of alternative IS481 primer sequences is unlikely to improve the specificity for *B. pertussis*.

The full clinical significance of false-positive *B. pertussis* IS481 PCR assay results due to detection of *B. holmesii* needs to be evaluated. Although *B. holmesii* is most often associated with septicemia in patients with underlying conditions (8, 9, 13, 20, 27), it recently accounted for <4% (32 of 868) of the *Bordetella* spp. cultured from nasopharyngeal secretions from Massachusetts patients with pertussis-like illness (12, 28). However, the clinical relevance and prevalence of *B. holmesii* in upper respiratory tract illness remain to be further investigated. Consequently, we recommend evaluation of other targets concurrent with the cautious use of IS481-based diagnostic PCR assays, including the interpretation and reporting of positive results.

**Nucleotide sequence accession number.** The *B. holmesii* IS481-like insertion sequence was deposited in GenBank (accession no. AF349431).

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