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Novel Duplex Real-Time PCR Assay Detects *Bordetella holmesii* in Specimens from Patients with Pertussis-Like Symptoms in Ontario, Canada▽

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***Bordetella holmesii* is a human pathogen found mainly in immunocompromised patients. A specific real-time PCR assay was developed and successfully used to identify specimens from which *B. holmesii* was misidentified as *Bordetella pertussis* and to establish the prevalence of *B. holmesii* in Ontario patients with pertussis-like symptoms.**

The Gram-negative bacterium *Bordetella holmesii* was first described in 1995. It is known to cause disease (e.g., septicemia) in patients with serious underlying medical conditions (2, 5, 7, 12, 14) and has also been detected in nasopharyngeal specimens from patients with a pertussis-like illness (6, 15). Two insertion sequences, IS*481* and IS*1001*, are commonly used PCR targets for the diagnosis of *Bordetella pertussis* and *Bordetella parapertussis*, respectively (4, 11). Since both targets are also present in *B. holmesii*, disease due to this pathogen may be mistakenly attributed to other *Bordetella* species (8, 9). The aim of this study was to develop a duplex real-time PCR assay able to detect and discriminate between *B. pertussis* and *B. holmesii*. Additionally, 1,775 specimens positive for IS*481* and/or IS*1001* collected during 2007 and 2008 were retrospectively tested with the new assay to determine the prevalence of *B. holmesii* in Ontario, Canada.

To date, the *B. holmesii* genome has not been fully sequenced, and thus, it is challenging to identify novel targets for specific PCR identification of this organism. The housekeeping gene *recA* has been used in previous studies for detection of *B. holmesii* (1, 13) and was selected as a PCR target for this study. Specific detection of *B. holmesii* was accomplished by designing a real-time PCR assay targeting a 50-bp segment of the *recA* gene (GenBank accession no. AF399664) that is polymorphic between *B. holmesii*, *B. pertussis*, *B. parapertussis*, *Bordetella bronchiseptica*, *Bordetella hinzii*, and *Bordetella avium* (Fig. 1).

To confirm the conservation of *B. holmesii recA* (BH*recA*) among clinical isolates, the full gene from 8 *B. holmesii* strains cultured from patients was amplified and sequenced. Sequencing revealed that BH*recA* was 100% conserved in the 8 isolates tested.

Analytical sensitivity of the assay was determined using a dilution series (10-fold dilutions, 5.5 ng to 5.5×10^{-10} ng) of BH*recA* cloned into pCR2.1 (Invitrogen, Carlsbad, CA). Each dilution was tested in triplicate, and the experiment was performed 3 separate times to ensure accuracy of the results. The assay found a 100% probability that detection of 55 ag of cloned material (equal to 2 copies of *recA*) was possible at a threshold cycle (C_T) of 38.3 ± 1.0. Furthermore, specificity of the real-time PCR assay was verified by testing purified genomic DNA from 32 different bacterial, fungal, and viral pathogens, including *B. pertussis* ($n = 54$), *B. parapertussis* ($n = 32$), *B. bronchiseptica* ($n = 1$), *B. hinzii* ($n = 1$), *Acinetobacter* sp., *Alcaligenes faecalis*, *Alcaligenes xylosoxidans*, *Achromobacter piechaudii*, *Corynebacterium diphtheriae*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus durans*, *Enterococcus hirae*, *Enterococcus gallinarum*, *Enterococcus avium*, *Enterococcus raffinosus*, *Escherichia coli*, *Haemophilus influenzae*, *Haemophilus parainfluenzae*, *Helicobacter pylori*, *Legionella pneumophila*, *Mycobacterium tuberculosis*, *Mycoplasma pneumoniae*, *Neisseria meningitidis* (A, B, C, W, X, Y, and Z), *Pseudomonas aeroginosa*, *Ralstonia eutropha*, *Ralstonia* sp., *Staphylococcus aureus*, *Streptococcus pyogenes*, *Candida albicans*, *Herpes simplex virus* 1, and *Herpes simplex virus* 2. The BH*recA* primers and probe successfully detected DNA from *B. holmesii* and not from any other *Bordetella* species or other pathogens tested.

To develop our novel duplex real-time PCR assay for the simultaneous detection of *B. pertussis* and *B. holmesii*, the BH*recA* primers and probe were combined with a currently used IS*481* real-time PCR. To ensure that sensitivity was not significantly affected by combining the targets, simplex IS*481* and simplex BH*recA* reactions were carried out and validated alongside the duplex assay. IS*481* real-time PCR was carried out using the published primers and probe of Kösters et al. (4), modified by using 3′ BHQ-1 for the probe (IDT, Coralville, IA). The oligonucleotide primers BHrecA_fwd (5′-CGGTTC GCTGGGTCTCG-3′) and BHrecA_rev (5′-CCCGCGGCAG ACCAC-3′) were used in this study for real-time PCR detection of *B. holmesii*. The TaqMan MGB probe was designed with a NED fluorescent reporter on the 5′ end and nonfluorescent black hole quencher at the 3′ end with the sequence 5′-CATCGCATTGGGCG-3′ (Applied Biosystems, Foster City, CA). All primers were purchased from Invitrogen.

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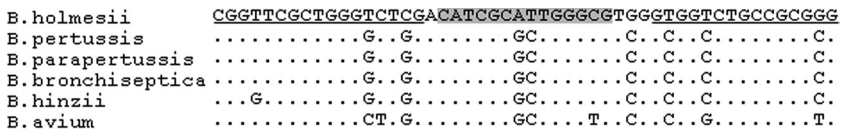

FIG. 1. Multiple sequence alignment of a 50-bp segment of the *recA* gene for several *Bordetella* species. Real-time PCR primers designed for this study are underlined, and the TaqMan MGB probe is highlighted.

The duplex PCR master mix consisted of 1× TaqMan gene expression master mix (Applied Biosystems), 0.8 μM (each) BH*recA* forward and reverse primers, 0.4 μM BH*recA* probe, 1 μM (each) IS*481* forward and reverse primers, 0.25 μM IS*481* probe, 5 μl of template, and enough sterile nuclease-free water to bring the total reaction volume to 20 μl. The simplex BH*recA* PCR master mix followed the same recipe with the elimination of IS*481* primers and probe. The currently used simplex IS*481* PCR master mix used 0.5 μM (each) IS*481* forward and reverse primers and 0.125 μM IS*481* probe. The samples were subjected to an initial amplification cycle of 50°C for 2 min and 95°C for 15 min, followed by 45 cycles at 94°C for 15 s and 60°C for 1 min. Five microliters of cloned BH*recA* at 5 fg/μl was used as a positive PCR control; the negative control was 5 μl of sterile H_2O. Amplification, detection, and data analysis were performed with an Applied Biosystems 7900HT real-time PCR system and the SDS v2.3 software program. A blinded set of 117 patient specimens, previously confirmed by culture, was used to validate the IS*481*/BH*recA* duplex PCR assay, with 100% concordance found in detecting *B. pertussis* and *B. holmesii*. It was found that the duplex values for IS*481* averaged 1.1 C_Ts lower than those for simplex IS*481* reactions. Furthermore, the duplex values for BHrecA were on average 2.4 C_Ts lower than those for simplex BHrecA reactions. Therefore, duplex reactions were more sensitive than simplex real-time PCR and could be used for routine diagnostics.

For detection of *B. holmesii* in clinical patient specimens, nasopharyngeal samples were obtained using Dacron tipped swabs (Bio Nuclear Diagnostics Inc., Toronto, CA) and transported in 500 μl of phosphate-buffered saline (PBS). Patient swabs from suspected *B. pertussis* cases were handled by the Respiratory & Legionnaires' section at the Central Public Health Laboratory (CPHL), where they were all processed for culture and then subjected to rapid boiling extractions for real-time PCR amplification using the targets IS*481* and IS*1001*, as well as an extraction and PCR control (human RNase P gene), the results of which can be seen in Table 1. PBS was found to have no inhibitory effect on the PCRs. A total of 1,775 *Bordetella*-positive patient specimens collected in 2007 and 2008 were retrospectively tested using the duplex real-time PCR assay to determine the prevalence of *Bordetella holmesii* in patients with suspected pertussis infections. All *B. holmesii* culture-positive specimens ($n = 8$) were positive using the real-time PCR duplex assay, and 4 additional specimens were identified as *B. holmesii* based on the duplex assay.

To further confirm that specimens positive for BH*recA* were not dual infections with *B. pertussis*, a second real-time PCR was run simultaneously using the BP3385 target, which is specific for *B. pertussis* (3). It was found that none of these BH*recA*-positive specimens were detected by BP3385.

Consistent with a study performed in the United States (15), the prevalence of *B. holmesii* in Ontario appears to be relatively low, with positivity in less than 1% of all nasopharyngeal swabs collected. However, *B. holmesii* is indeed present and associated with pertussis-like symptoms in patients. A key point for clinicians and public health workers elucidated in this study is that use of IS*481* for the detection of *B. pertussis* should be done with the awareness of its cross-reactivity, since four previously misidentified specimens were detected using the *B. holmesii*-specific real-time PCR assay. At present, the clinical impact of misdiagnosing a *B. holmesii* infection as *B. pertussis* is unknown. The limited data available indicate *B. holmesii* is susceptible to a number of antibiotics (5, 12). However, it has not been established if erythromycin, the most commonly used drug for treatment of pertussis, is effective against *B. holmesii*, and MIC testing has suggested that erythromycin is less active against *B. holmesii* than against *B. pertussis* (10). Therefore, correct identification of this pathogen is important for active

TABLE 1. Results of real-time PCR using BH*recA*, IS*481*, and IS*1001* for patient specimens collected over a 2-year period[a]

Target	Organism detected	2007 specimens			2008 specimens		
		No. of specimens	% of total *Bordetella*-positive specimens	% of total specimens	No. of specimens	% of total *Bordetella*-positive specimens	% of total specimens
IS*481*	*B. pertussis*	883	95.4	17.7	824	97.1	15.6
IS*1001*	*B. parapertussis*	26	2.8	0.5	18	2.1	0.3
IS*481* and IS*1001*	*B. pertussis* and *B. parapertussis*[b]	8	0.9	0.2	4	0.5	0.5
IS*481* and BH*recA*	*B. holmesii*	9[c]	1.0	0.2	3[d]	0.4	0.1
Total *Bordetella* positive		926		18.6	849		16.5
Total specimens received		4,983			5,271		

[a] *Bordetella* species were differentiated based on the detection of IS*481* without detection of BH*recA* (*B. pertussis*) or detection of both IS*481* and BH*recA* (*B. holmesii*).
[b] Coinfection.
[c] Six were culture positive.
[d] Two were culture positive.

surveillance of *Bordetella holmesii* as an infectious agent and for greater determination of its role as a respiratory pathogen.

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