Genetic diagnosis and molecular epidemiology of *Bordetella*

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Summary:

Bordetella holmesii infection has never been reported in Taiwan. In current surveillance study, only one case of possible *B. holmesii* infection was found among 653 notified cases of pertussis (0.15%) in 2011 - 2013. This possible case was a 12-year-old boy who was notified in May 2011. According to this surveillance results, the prevalence of *B. holmesii* infection in Taiwan was extremely low among patients who had pertussis-like symptoms and were notified. Based on its high sensitivity, the IS*481*-based PCR was routinely used worldwide by many laboratories, however, it should be done with the awareness of its cross-reactivity. *B. holmesii* has been more and more frequently detected in biological samples from adolescents and adults of pertussis-like symptoms. Although differentiation might not be necessary for treatment, it is valuable in epidemiological settings to distinguish among *Bordetella* species. Improved specificity of detection would provide us an insight into the real burdens of *B. pertussis* and *B. holmesii* infection, and information regarding vaccine failure due to misdiagnosis and possible response strategy. In conclusion, surveillance of *B. holmesii* should be pursued, and correct identification of *Bordetella* species is important for active surveillance of *Bordetella* infections in the whole population, particularly in adolescents and adults.

I. Purpose:

In previous surveillance studies conducted in the USA and Canada, a low positive rate for *B. holmesii* infection (0.1–0.3%) was reported by culture or RT-PCR in patients with coughs (1, 2). In a recent study in France, however, *B. holmesii* DNA was detected in 20% of nasopharyngeal swabs (NPSs) collected from adolescent patients who had previously been diagnosed with *B. pertussis* infection (3). Furthermore, between 2010 and 2011, a pertussis outbreak caused by *B. pertussis* and *B. holmesii* infections occurred in Miyazaki Prefecture, Japan (4). These surveillance data indicated that *B. holmesii* infection has recently spread worldwide and that

accurate diagnosis is needed to distinguish between *B. holmesii* and *B. pertussis* infections. Therefore, the purpose of this surveillance study is to detect *B. holmesii* in NPSs received in our laboratory from all cases who had pertussis-like symptoms and were notified in 2011 -2013.

II. Methods:

We applied *B. pertussis*-LAMP assay and a novel duplex real-time PCR assay to NPSs from 653 patients who had pertussis-like symptoms and were notified in 2011 - 2013. These patients were collected from Taiwan pertussis notified disease surveillance system. We obtained the NPSs using ESwab™ Nylon Flocked Swab and 1mL of modified Liquid Amies (Copan).

B. pertussis-LAMP assay (detection of *ptxP*): A 25μL reaction mixture containing 40 pmol (each) of BP-FIP and BP-BIP primers, 5 pmol (each) of BP-F3 and BP-B3 primers, 20 pmol (each) of BP-LF and BP-LB primers, 2X reaction mixture (12.5 μL), *Bst* DNA polymerase (1 μL), and template DNA (2 μL) was used. The mixture was incubated at 65°C for 40 min (for clinical specimens) and then heated at 80°C for 2 min to terminate the reaction. All oligonucleotides (high-performance liquid chromatography purification grade) for the LAMP primers were obtained from Invitrogen Taiwan Ltd. The LAMP amplification was confirmed with real-time monitoring of the increase of turbidity using LA-320C (Eiken Chemical Co., Ltd.) (5).

Novel duplex real-time PCR assay (detection of IS*481* and BH*recA*): The duplex PCR master mix consisted of 1X Premix master mix (Premix EX Taq, RR039A, Takara), 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, 2 μL of template DNA, and enough sterile nuclease-free water to bring the total reaction volume to 20 μL. The samples were subjected to an initial amplification cycle of 95°C for 30s, followed by 40 cycles at 95°C for 5s and 60°C for 34s. Two microliters of *B. holmesii* ATCC51541 (500 pg/μL) with 6 series of 10-fold dilution was used as a positive PCR control; the negative control was 2 μL of sterile H_2O. Amplification, detection, and data analysis were performed with an Applied Biosystems 7500 real-time PCR system and the 7500 software v2.0 (1).

III. Results:

1) Results of real-time PCR using IS*481*, BH*recA* and *ptxP* (LAMP) for NPSs in 2011-2013

Target	Target organism	2011		2012		2013	
		No. of specimens	% of total specimens	No. of specimens	% of total specimens	No. of specimens	% of total specimens
IS*481* without BH*recA*	*B. pertussis*	60	16.7	32	22.4	34	22.6
IS*481* and BH*recA*	*B. holmesii*	1	0.3	0	0	0	0
ptxP (LAMP)	*B. pertussis*	47	13.1	27	18.9	32	21.2
Total specimens tesed		359		143		151	

B. holmesii DNA was detected only in one case notified in May 2011. This case was a 12-year-old boy. Positive rate of *B. pertussis* using IS*481*-based PCR was 16.7%, 22.4% , 22.6% in 2011-2013, respectively. Positive rate of *B. pertussis* using *ptxP*-based PCR was 13.1% , 18.9%, 21.2% in 2011 - 2013, respectively.

2) An investigation of a school outbreak of pertussis

In December 2013, a suspected outbreak of pertussis occurred in a middle school in Longtan, Taoyuan County. The index case was a 13-year-old boy. He started with flu-like symptoms on November 10, and went to the En Chu Kong Hospital for medical service on December 10 due to persistent symptoms. He was notified as a case with pertussis. He was tested positive for pertussis by PCR on December 17, and by bacterial culture on December 19. A school clustering of pertussis was found through the System of Epidemiological Investigation at the Centers for Disease Control. Nasopharyngeal swabs were obtained from 8 contacts who developed pertussis-like symptoms between November 27 and December 9. Four contacts were tested positive for pertussis by *ptx*P-based PCR, and IS*481* without HB*recA* real-time PCR on December 20, and later by bacterial culture.

IV. Discussion:

Surveillance result of *B. holmesii* infection in Taiwan revealed that there was only one possible case, a 12-year-old boy, in May 2011. This result indicated that the prevalence of *B. holmesii* infection in Taiwan was very low among patients who had pertussis-like symptoms and were notified. Whether this case was a real case of *B. holmesii* infection was not certain. Although the duplex real-time PCR gave a positive result, sequencing of the DNA product was not successful because the *B. holmesii* DNA content in the specimen was too low.

The positive rate of diagnosis was 50% among the investigation of the school outbreak of pertussis, revealing that pertussis is a highly contagious disease. Following the confirmation of the index case, all contacts with pertussis-like symptoms received antimicrobial agents for 5 days. No more cases were recognized then, indicating that rapid, accurate diagnosis combined with correct treatment and prevention will greatly aid in disease control. The duplex real-time PCR is the method that can provide timely and accurate diagnosis to reveal pathogens that cause the infections.

From studies worldwide, most cases from whom *B. holmesii* was detected were adolescents and adults, especially significant occurrence in adolescents, but not in infants. However, the age distribution of our cases was 55.1% , 48.5% , 68.9% among infants less than 1 year old in 2011 - 2013, respectively, and 12.7% , 12.5%, 9.9% among children aged 10-19 years in 2011 - 2013, respectively. There might not be sufficient specimens from adolescents in our study, thus, leading to low prevalence. Nevertheless, *B. holmesii* was indeed present and associated with pertussis-like symptoms in patients, indicating that surveillance of *B. holmesii* infection is important.

Based on its high sensitivity, the IS*481*-based PCR was routinely used worldwide by many laboratories, however, it should be done with the awareness of its cross-reactivity. *B. holmesii* has been more and more frequently detected in biological samples from adolescents and adults of pertussis-like symptoms. Although differentiation might not be necessary for treatment, it is valuable in epidemiological settings to distinguish among *Bordetella* species. Improved specificity would advance our understanding of burdens from *B. pertussis* and *B. holmesii*, reduce concerns arising from apparent vaccine failures due to misdiagnosis, and might provide information on which vaccine-based outbreak response strategies can be based (6). In conclusion, surveillance of *B. holmesii* should be pursued and correct identification of *Bordetella* species is important for active surveillance of *Bordetella* infections in the whole population, particularly in adolescents and adults.

V. Reference list:

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VI. Publication list for this work:

Nil