ORIGINAL ARTICLE

Laboratory-based surveillance of pertussis using multitarget real-time PCR in Japan: evidence for *Bordetella pertussis* infection in preteens and teens

K. Kamachi[1], S. Yoshino[2], C. Katsukawa[3], N. Otsuka[1], Y. Hiramatsu[1] and K. Shibayama[1]

1) *Department of Bacteriology II, National Institute of Infectious Disease, Tokyo,* 2) *Department of Microbiology, Miyazaki Prefectural Institute for Public Health and Environment, Miyazaki and* 3) *Department of Infectious Diseases, Osaka Prefectural Institute of Public Health, Osaka, Japan*

Abstract

Between January 2013 and December 2014, we conducted laboratory-based surveillance of pertussis using multitarget real-time PCR, which discriminates among *Bordetella pertussis, Bordetella parapertussis, Bordetella holmesii* and *Mycoplasma pneumoniae*. Of 355 patients clinically diagnosed with pertussis in Japan, *B. pertussis, B. parapertussis* and *M. pneumoniae* were detected in 26% (n = 94), 1.1% (n = 4) and 0.6% (n = 2), respectively, whereas *B. holmesii* was not detected. It was confirmed that *B. parapertussis* and *M. pneumoniae* are also responsible for causing pertussis-like illness. The positive rates for *B. pertussis* ranged from 16% to 49%, depending on age. Infants aged $\leq$ 3 months had the highest rate (49%), and children aged 1 to 4 years had the lowest rate (16%, $p < 0.01$ vs. infants aged $\leq$ 3 months). Persons aged 10 to 14 and 15 to 19 years also showed high positive rates (29% each); the positive rates were not statistically significant compared with that of infants aged $\leq$ 3 months ($p \geq 0.06$). Our observations indicate that similar to infants, preteens and teens are at high risk of *B. pertussis* infection.

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Keywords: *Bordetella holmesii*, *Bordetella parapertussis*, *Bordetella pertussis*, *Mycoplasma pneumoniae*, pertussis, real-time PCR
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Corresponding author: K. Kamachi, Department of Bacteriology II, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan
E-mail: kamachi@nih.go.jp

Introduction

Pertussis (whooping cough) is a major acute respiratory infection caused by *Bordetella pertussis* bacteria and is associated with severe respiratory illness in children and persistent cough in adolescents and adults. The most effective method to prevent and control pertussis is immunization; however, the incidence of pertussis has increased in several developed countries despite high vaccination coverage [1]. *Bordetella parapertussis* and *Bordetella holmesii*, which are closely related to *B. pertussis*, also cause pertussis-like coughs [2,3]. Acellular pertussis vaccines (ACVs) have little or no protection against *B. parapertussis* and *B. holmesii* infections, both of which have been detected recently with significant percentages in pertussis-like illness [4–6]. In addition, *Mycoplasma pneumoniae* is a bacterial agent of atypical pneumonia in children and adults that also causes a persistent pertussis-like cough [7]. The clinical diagnosis of *B. pertussis* infection is complicated by other respiratory pathogens [8].

In Japan, the incidence of pertussis cases in adolescents and adults has significantly increased since the early 2000s, and a large pertussis epidemic occurred between 2008 and 2010 despite high vaccination coverage with ACVs [9]. A total of 17 349 cases were reported from approximately 3000 sentinel clinics and hospitals in the epidemic. The national pertussis surveillance data regarding individuals diagnosed mainly on the basis of clinical symptoms, bacterial culture and/or serologic testing are collected. In comparison to culture and serology, which are the classic tests for pertussis diagnosis, nucleic acid amplification tests have improved sensitivity and specificity; however, these tests have not yet been widely introduced into

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Japan. Therefore, our laboratory, a national reference laboratory for pertussis, developed a multitarget real-time PCR assay (4Plex RT-PCR) to discriminate among *B. pertussis, B. parapertussis, B. holmesii* and *M. pneumoniae* and then introduced the assay into our diagnostic service starting in January 2013. The present study reports 2 years of experience of the service for 355 patients with clinically suspected pertussis.

Materials and Methods

Clinical specimens

Between January 2013 and December 2014, we conducted a laboratory-based surveillance study of 355 patients clinically diagnosed with pertussis. Clinical specimens (nasopharyngeal swab or aspirate) were collected at 19 medical institutions in Japan. All the nasopharyngeal aspirates (2% of the total number of specimens) were collected from young infants hospitalized with severe respiratory distress. The specimens were transported to the National Institute of Infectious Diseases, Japan. Nasopharyngeal swabs were immersed in 0.5 mL of saline, vortexed and subjected to centrifugation (20 000 × *g* for 10 minutes). Nasopharyngeal aspirates (50–100 μL) were suspended in 1 mL of saline, vortexed, and centrifuged at the same conditions. Total DNA was extracted from the pellet using the QIAamp DNA Micro kit (Qiagen) and eluted with 25 μL of the AE elution buffer. If several specimens were received from a single patient, they were tested separately. The overall result was considered positive if any of these specimens tested positive.

In Japan, pertussis cases are reported based on clinical diagnosis. The clinical criteria are cough lasting for $\geq$ 2 weeks with one or more of the following symptoms: whoop and staccato cough, apneic paroxysm or posttussive vomiting. However, in the present study, patients were clinically diagnosed based on their physician's judgement, so not all patients met the reporting criteria.

This study was considered exempt from institutional review board approval because clinical specimens were obtained for diagnostic and surveillance purposes.

4Plex RT-PCR

Target sequences of the 4Plex RT-PCR were insertion sequence IS*481* (detection for *B. pertussis* and *B. holmesii*), *recA* (*B. holmesii*), IS*1001* (*B. parapertussis*) and *atpD* (*M. pneumoniae*). Published primers and probes were used for *recA* and *atpD* [10,11], and those for IS*481* and IS*1001* were used with minor modifications [12] (Table 1). 4Plex RT-PCR was performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems). The PCR amplifications were carried out in 20 μL reaction volumes containing 10 μL of 2× *Premix EX Taq* (Perfect Real Time, Takara Bio), 0.2 μL of 50× ROX reference dye II, 2 μL of DNA samples and optimized concentrations of primers and probes (Table 1). The PCR conditions were 10 s at 95°C, followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. The cutoff threshold ΔRn (relative fluorescent intensity) value was set to 0.3 for all fluorescence signals, and the sample was considered positive with a change in ΔRn of $\geq$ 0.3, corresponding to a threshold cycle (C_T) cutoff value of approximately 36 with automatic calculation. In each assay, purified DNAs (10 pg of *B. pertussis* Tohama, 100 pg of *B. holmesii* ATCC51542, 10 pg of *B. parapertussis* BAA-587, 1 pg of *M. pneumoniae* NBRC14401) were used as positive controls, whereas the negative control was sterile distilled water. The analytical sensitivities of the assay were not affected by multiplexing (Supplemental Fig. S1). The measured PCR amplification efficiencies of 98% to 103% for the targets *B. holmesii* IS*481* and *recA* and *M. pneumoniae atpD* were in good agreement with the

TABLE 1. Primers and probes used for 4Plex real-time PCR

Target gene (organism)	Primer or probe	Sequence (5′ to 3′)	Reporter/ quencher	Amplicon (bp)	Optimal concentration (nM)	Reference
IS*481* (*Bordetella pertussis* and *Bordetella holmesii*)	PPertM	ATCAAGCACCGCTTTACCCG[a]		114	300	[12]
	APPert	TTGGGAGTTCTGGTAGGTGTG			300	
	SPertM	CAAGGCCGAACGCTT[a]	FAM/NFQ-MGB[b]		200	
recA (*B. holmesii*)	BHrecA-F	CGGTTCGCTGGGTCTCG		50	400	[10]
	BHrecA-R	CCCGCGGCAGACCAC			400	
	BHrecA-P	CATCGCATTGGGCG	VIC/NFQ-MGB		300	
IS*1001* (*B. parapertussis*)	PParaP	GATATCAACGGGTGACGGATC		103	300	[12]
	APParaP	GTATGCCAACCCAGTTCGAA			300	
	SParaM	TGCAATCGAGCAACG[a]	NED/NFQ-MGB		100	
atpD (*M. pneumoniae*)	Mp3-F	CGATCTATGTGCCAGCTGATGA		68	200	[11]
	Mp3-R	AGCATCCAGGTGGGTAAAGGT			200	
	Mp3-P	TTGACTGACCCCGCTCCGGC	Cy5/BHQ3[c]		100	

[a]Oligonucleotide length was modified.
[b]Non-Fluorescent Quencher-Minor Groove Binder.
[c]Black Hole Quencher 3.

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