

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

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## 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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## 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

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 ≥ 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 IS481 (detection for *B. pertussis* and *B. holmesii*), *recA* (*B. holmesii*), IS1001 (*B. parapertussis*) and *atpD* (*M. pneumoniae*). Published primers and probes were used for *recA* and *atpD* [10,11], and those for IS481 and IS1001 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 ≥ 0.3, corresponding to a threshold cycle (C<sub>T</sub>) 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* IS481 and *recA* and *M. pneumoniae atpD* were in good agreement with the

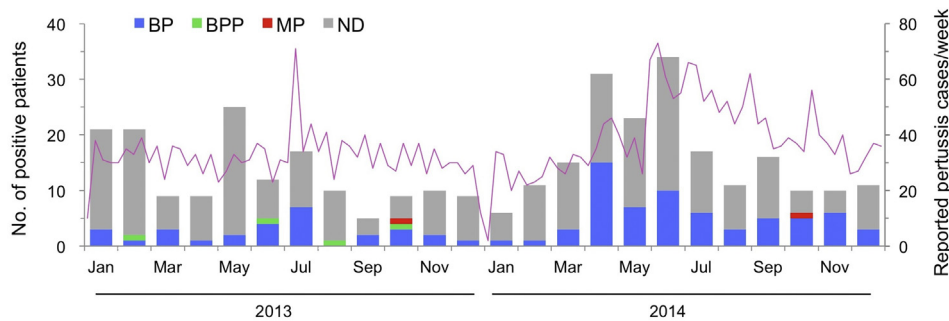
TABLE 1. Primers and probes used for 4Plex real-time PCR

Target gene (organism)	Primer or probe	Sequence (5' to 3')	Reporter/ quencher	Amplicon (bp)	Optimal concentration (nM)	Reference
IS481 ( <i>Bordetella pertussis</i> and <i>Bordetella holmesii</i> )	PPertM	ATCAAGCACCGCTTTACCCG <sup>a</sup>	FAM/NFQ-MGB <sup>b</sup>	114	300	[12]
	APPert	TTGGGAGTTCTGGTAGGTGTG			300	
	SPertM	CAAGGCCGAAACGCTT <sup>c</sup>			200	
<i>recA</i> ( <i>B. holmesii</i> )	BHrecA-F	CGGTTCCGCTGGGTCTCG	VIC/NFQ-MGB	50	400	[10]
	BHrecA-R	CCCGCGGCAGACCAC			400	
	BHrecA-P	CATCGCATTGGGCG			300	
IS1001 ( <i>B. parapertussis</i> )	PParaP	GATATCAACGGGTGACGGATC	NED/NFQ-MGB	103	300	[12]
	APParaP	GTATGCCAACCCAGTTCGAA			300	
	SParaM	TGCAATCGAGCAACG <sup>a</sup>			100	
<i>atpD</i> ( <i>M. pneumoniae</i> )	Mp3-F	CGATCTATGTGCCAGCTGATGA	Cy5/BHQ3 <sup>c</sup>	68	200	[11]
	Mp3-R	AGCATCCAGGTGGGTAAAGGT			200	
	Mp3-P	TTGACTGACCCCGCTCCGGC			100	

<sup>a</sup>Oligonucleotide length was modified.

<sup>b</sup>Non-Fluorescent Quencher-Minor Groove Binder.

<sup>c</sup>Black Hole Quencher 3.



**FIG. 1.** Monthly distribution of samples positive for *Bordetella pertussis* (BP), *Bordetella parapertussis* (BPP) and *Mycoplasma pneumoniae* (MP) between January 2013 and December 2014 in Japan. A total of 355 patients were tested using 4Plex RT-PCR. Numbers of patients with positive and negative results are shown by vertical bars (left y-axis). No samples were positive for *Bordetella holmesii*. Reported pertussis cases in Japan (national pertussis surveillance data) are shown by purple line (right y-axis), with each value representing a week of the year. National surveillance data were obtained from Infectious Disease Surveillance data of the Ministry of Health, Labor and Welfare of Japan. ND, not detected for any of the organisms.

theoretical value of 100%. The efficiencies for *B. pertussis* IS481 and *B. parapertussis* IS1001 showed somewhat higher values (116–117%), the reason for which is currently unclear.

In the 4Plex RT-PCR assay, *B. pertussis* infection was defined as positive for IS481 and negative for *recA* (IS481<sup>+</sup>/*recA*<sup>-</sup>), whereas *B. holmesii* was defined as positive for both IS481 and *recA* (IS481<sup>+</sup>/*recA*<sup>+</sup>). *B. parapertussis* and *M. pneumoniae* were defined as positive for IS1001 and *atpD*, respectively.

### Statistical analysis

Data were analysed by Fisher's exact test. A value of  $p < 0.05$  was considered statistically significant.

## Results

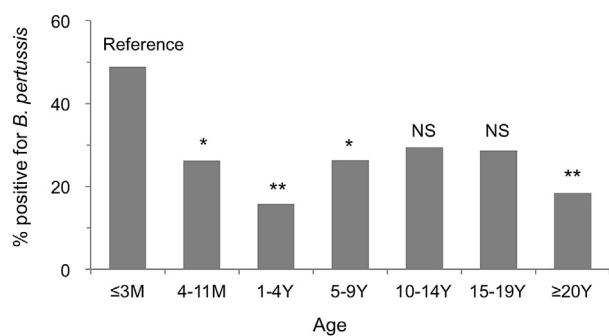
Among 355 patient samples, 94 (26%), four (1.1%) and two (0.6%) were positive for *B. pertussis*, *B. parapertussis* and *M. pneumoniae*, respectively. There was no incidence of coinfection with those organisms. No positive samples for *B. holmesii* were detected, but 4Plex RT-PCR targeting *recA* is less sensitive

than IS481. Fig. 1 shows the monthly distribution of samples testing positive using PCR. *B. pertussis*-positive samples were observed in relatively large numbers from April to June 2014 (34% of the total) and also showed a tendency to decrease in the winter season from December to February. Four *B. parapertussis*-positive samples were detected in 2013. All of them were collected in the same hospital, with no apparent seasonal variations. Two *M. pneumoniae*-positive samples were detected in October 2013 and 2014, and confirmed using a separate genetic test (Loopamp Mycoplasma P Detection Kit; Eiken Chemical, Japan). Our pertussis surveillance data did not fully correspond with national pertussis surveillance data, especially in the period from April to August 2014.

As shown in Table 2, of 94 *B. pertussis*-positive samples, 21 were from infants aged  $\leq 3$  months, and 20 were from children aged 5 to 9 years. Of four *B. parapertussis*-positive samples, three and one were from children aged 1 to 4 years and 5 to 9 years, respectively. Similarly, two *M. pneumoniae*-positive samples belonged to the same age groups. Fig. 2 shows positive rates for *B. pertussis* by age group. Infants aged  $\leq 3$  months had the highest rate (49%), whereas children aged 1 to 4 years had

**TABLE 2.** Results of 4Plex real-time PCR for 355 patient samples collected between 2013 and 2014

Patient age range	No. of samples tested	No. of positive results			
		<i>Bordetella pertussis</i>	<i>Bordetella parapertussis</i>	<i>Bordetella holmesii</i>	<i>Mycoplasma pneumoniae</i>
< 3 months	43	21	–	–	–
4–11 months	42	11	–	–	–
1–4 years	70	11	3	–	1
5–9 years	76	20	1	–	1
10–14 years	51	15	–	–	–
15–19 years	21	6	–	–	–
$\geq 20$ years	49	9	–	–	–
Unknown	3	1	–	–	–
Total	355	94 (26%)	4 (1.1%)	0	2 (0.6%)



**FIG. 2.** Age distribution of PCR-positive rates for *Bordetella pertussis*. Total of 93 patients of known age were classified into seven age groups (Table 2). Statistically significant differences (Fisher's exact test) between infants aged  $\leq 3$  months (reference) and other age groups are indicated by asterisks (\* $p < 0.05$ ; \*\* $p < 0.01$ ). Age groups not significantly different from reference group are indicated by NS. M, months; Y, years.

the lowest rate (16%). Of note, the positive rates showed a weak bimodal distribution by age group. The positive rates of the age groups 4 to 11 months, 1 to 4 years, 5 to 9 years, and  $\geq 20$  years were statistically lower than that of infants aged  $\leq 3$  months ( $p < 0.05$ , Fisher's exact test). In contrast, the positive rates of the age groups 10 to 14 and 15 to 19 years were not statistically significant with that of infants aged  $\leq 3$  months ( $p$  0.06 and 0.18, respectively).

## Discussion

This study confirmed that *B. pertussis* is the most common aetiologic agent in cases of suspected pertussis and that *B. parapertussis* and *M. pneumoniae* are also responsible for causing a small proportion of pertussis-like illnesses. In this study, no positive samples for *B. holmesii* were detected, although respiratory infection with *B. holmesii* has recently increased worldwide [3,5,6]. 4Plex RT-PCR has high analytical sensitivities for *B. pertussis*, *B. parapertussis* and *M. pneumoniae* (detection limits of 10–100 fg genomic DNA), but it has a relatively low sensitivity for *B. holmesii recA* (detection limit of 1 pg DNA, corresponding to a  $C_T$  value of 35.6). We therefore cannot exclude the possibility that some patients could have low *B. holmesii* DNA loads ( $C_T$  value of  $> 35.6$ ) in their clinical specimens. However, we previously reported that patients not treated with antibiotics had high *B. holmesii* DNA loads ( $C_T$  values of 21.6 to 28.7) in their nasopharyngeal swab samples [5]. Thus, 4Plex RT-PCR has sufficient sensitivity to detect *B. holmesii* in clinical specimens. A recent study has found that an increasing number of *B. holmesii* bacteraemia cases were associated with outbreaks of *B. pertussis* [13]. This suggests that

*B. holmesii* epidemics occur periodically, similar to *B. pertussis*. In Japan, a *B. pertussis* epidemic has not occurred from 2011 to the present; hence, continuous laboratory-based surveillance is required for *B. holmesii*.

In the present study, *B. pertussis*-positive samples were detected from individuals over a wide age range, consistent with previous reports [14–17]. Infants aged  $\leq 3$  months had the highest positive rate (49%); interestingly, persons aged 10 to 14 and 15 to 19 years also showed high positive rates (each 29%). In Japan, ACVs are used to control pertussis with a schedule of three primary doses and a single booster dose at ages 3, 4, 6 and 18 to 23 months, respectively. The vaccination is done under the Japanese Preventive Vaccination Law, and consequently immunization rates among children are high ( $\geq 90\%$ ). The duration of immunity after vaccination is estimated to be in the range of 4 to 12 years [18,19]; accordingly, preteens and teens are considered to be at high risk of *B. pertussis* infection. Our data provide support for the waning immunity in the second decade of life. Moreover, this study also demonstrates that children aged 1 to 4 years had the lowest positive rate (16%). This age group covers children from the age where they have received at least three doses of ACVs. The herd immunity against *B. pertussis* would be well maintained in the age group of 1 to 4 years. The lack of detailed clinical information (e.g., duration of cough, time of specimen collection and time of onset of symptoms) makes it difficult to explain why the *B. pertussis*-positive rate is equally low (18%) in the  $\geq 20$  years age group.

Recent genomic analyses revealed that some *Bordetella bronchiseptica* isolates (a broad-host-range pathogen) harbor IS481 and/or IS1001 [20]. This implies that *B. bronchiseptica* cannot be discriminated from *B. pertussis* and *B. parapertussis* using 4Plex RT-PCR. However, *B. bronchiseptica* infection in humans is rare [21,22]; are only two case reports exist from Japan. Therefore, we believe that *B. bronchiseptica* infection is negligible in our results.

In conclusion, we successfully used 4Plex RT-PCR for the surveillance of pertussis in Japan. Additionally, we confirmed that, similar to infants, preteens and teens are at high risk of *B. pertussis* infection. The 4Plex RT-PCR described here has become a practical tool for detection of pertussis, and therefore we have started supplying the 4Plex RT-PCR kit to Japanese prefectural and municipal public institutes for regional surveillance.

## Acknowledgements

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.nmni.2015.10.001>.

## Conflict of Interest

None declared.

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