and sensitive diagnosis, but also for quantitative analysis. The IS*481* real-time PCR can detect other *Bordetella* subspecies, such as *Bordetella holmesii*, *Bordetella bronchiseptica* and *Bordetella parapertussis*, although this assay is now widely used for the diagnosis of *B. pertussis* [14,15]. In neonates and young children with pertussis, the IS*481* real-time PCR showed that the *B. pertussis* DNA load in nasopharyngeal swabs (NPS) persists for a long time (3 weeks) after administration of antimicrobials [16,17]. For other pathogenic agents causing respiratory tract infections, such as *Moraxella catarrhalis* and *Streptcoccus pneumoniae*, the quantitative changes in bacterial DNA load in NPS correlate with the numbers of organisms detected by semiquantitative culture [18,19].

The present study aimed to determine *B. pertussis* DNA loads in NPS among adults and children, who were confirmed to have pertussis by *B. pertussis*-specific LAMP assay, by using quantitative IS*481* real-time PCR. We also investigated the relationship between *B. pertussis* load and bacterial genotypes.

Materials and Methods

Clinical samples

Approximately 200 NPS were obtained from adults (≥16 years old) and children (≤15 years old) with suspected pertussis between June 2007 and September 2009. The NPS were collected with sterilized rayon-tipped swabs (Eiken Chemical Co., Ltd, Tokyo, Japan) and then transported to the National Institute of Infectious Diseases, Japan. NPS were immersed in 0.5 mL of saline, vortexed, and precipitated by centrifugation (20 000 g for 10 min). Total DNA was extracted from the precipitation using QIAamp DNA Micro-kit (Qiagen, Hilden, Germany), and eluted with 25 μL of the AE elution buffer. The DNA samples were stored at −20°C until used.

LAMP assay

To confirm *B. pertussis* infection, a *B. pertussis*-specific LAMP assay was performed on DNA samples from NPS as described previously [11]. The LAMP amplification was performed with a 60-min reaction, and confirmed with real-time monitoring of the increase in turbidity using LA-320C (Eiken Chemical Co., Ltd). Among the DNA samples tested, 19 from adults and 40 from children (14 infants and 26 older children) were positive in the LAMP assay (≥0.1 turbidity), respectively. The vaccination history was obtained in 13 infants and 12 older children, but not in adults. Of 13 infants, 11 (85%) had never been immunized with pertussis vaccine. By contrast, ten (83%) of 12 older children had received four doses of pertussis vaccine.

IS*481* real-time PCR

Quantitative real-time PCR targeting IS*481* was performed by an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) as described previously [12], with minor modifications. Briefly, the IS*481* real-time PCR amplifications were done in 20-μL reaction volumes containing 1× *Premix Ex Taq*™ (Perfect Real Time; Takara Bio Inc., Shiga, Japan), 0.9 μM primer PPert and APPert, 0.25 μM TaqMan probe SPert, 0.4 μL of 50× Rox reference dye II, and 2 μL of DNA sample. The PCR conditions were 15 s at 95°C, followed by 40 cycles of 95°C for 3 s and 57°C for 30 s. Real-time data were analyzed by Sequence Detection Systems software, version 1.4 (Applied Biosystems). Standard curve was generated with ten-fold serial dilutions of *B. pertussis* Tohama DNA from 10 ng (2.4×10^6 bacterial cells) to 0.1 fg (0.024 bacterial cells). The number of bacterial cells was calculated with the Tohama genome size of 4.1 Mbp (2.4 genomic copies/10 fg DNA) [20].

The sampling efficiency of NPS and the presence of PCR inhibitors were examined by amplification of the human β_2-microglobulin gene with primers B2M-TR-1 and B2M-TR-2 [16] using SYBR green-based real-time PCR. Each 12 adult and child DNA samples, which were randomly selected from LAMP-positive samples, showed β_2-microglobulin C_t values in the range 15.1–24.8 (mean 21.9) and 19.8–22.3 (mean 21.1), respectively, confirming that the respective sampling efficiency and PCR inhibition were almost identical between adult and child NPS samples.

Multilocus sequence typing (MLST)

B. pertussis allelic genes (pertussis toxin *ptxA*, pertactin *prn* and serotype 3 fimbriae *fim3*) were amplified directly from patient DNA samples by nested-PCR. The first PCR was performed in a 15-μL reaction volume containing 1.87 μL DNA sample, 0.44 mM concentrations of each dNTPs, 0.2 μM concentrations of each primer, and 0.3 U of KOD-FX DNA polymerase (Toyobo, Osaka, Japan). For the nested-PCR amplification, 1 μL of the first PCR product was added as the template to the PCR mixture containing the same components described above, except that the nested primers were used instead of the first set of primers (Table 1). Cycling conditions were: denaturation for 1 min at 94°C; 30 cycles of 10 s at 98°C, 30 s at 55°C, 45 s at 68°C; and a postextension of 5 min at 72°C. The PCR products were sequenced with the BigDye terminator v3.1 cycle sequencing kit on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The combined allelic profiles were used to define MLSTs as described previously [2].

TABLE 1. Primers used in multilocus sequence typing analysis

Target gene	Primer name	Sequence (5′- to 3′)	PCR	Coordinate[a]
ptxA	ptx-outerF	TAACGCGGGTCTATCACAAC	1st	3988790
	ptx-outerR	TAGAACGAATACGCGATGCT	1st	3989047
	ptx-innerF	GACCACGACCACGGAGTATT	2nd	3988824
	ptx-innerR	GTACACGAGAACCATCGCCT	2nd	3989021
prn	prn-AF	GCCAATGTCACGGTCCAA	1st	1098595
	prn-AR	GCAAGGTGATCGACAGGG	1st	1099163
	prn-innerF	GTCATTGCAGCCGGAAGACC	2nd	1098657
	prn-innerR	CCGGTCTCGATGACATTGCC	2nd	1099111
fim3	Fim3-F1	ATGTCCAAGTTTTCATACCC	1st	1647602
	Fim3-R1	GGTGACCTTGCCGGTAAA	1st	1648082
	fim3-innerF	CCAGCACCCTCAACCATATC	2nd	1647738
	fim3-innerR	GGCTTGCGTGGTTTTGTC	2nd	1648055

[a]Coordinates in *Bordetella pertussis* Tohama genome sequence NC02929.

Statistical analysis

Data were analyzed using the Mann–Whitney *U*-test. $p < 0.05$ was considered statistically significant.

Results

Sensitivity and specificity of IS*481* real-time PCR

The real-time PCR with ten-fold serial dilutions (0.1 fg to 10 ng) of *B. pertussis* Tohama DNA was able to detect bacterial DNA over a linear range between 10 fg (2.4 bacterial cells) and 10 ng (2.4×10^6 bacterial cells) per reaction mixture ($r^2 = 0.99$) (Fig. 1). On the basis of three independent experiments, the detection limit was a threshold cycle (C_t) of 37.6 ± 0.3, corresponding to 2.4 cells of the Tohama. The analytical sensitivity of the IS*481* real-time PCR was equal to that of the *B. pertussis*-specific LAMP assay [11].

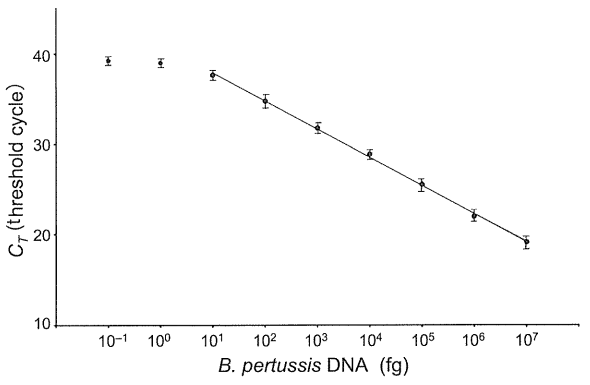

FIG. 1. Detection limit for linear calibration of IS*481* real-time PCR. Serial dilutions of *Bordetella pertussis* Tohama DNA were subjected to the real-time PCR. Data are the mean ± SD for three independent experiments.

Fifty-nine LAMP-positive (19 adults and 40 children) and 24 LAMP-negative DNA samples (six adults and 18 children, randomly selected) were subjected to the IS*481* real-time PCR. The LAMP-positive samples had C_t values in the range 14.6–39.5 (mean 29.7). By contrast, when the real-time PCR assay was applied to the LAMP-negative samples, the C_t values were in the range 37.2–40 (mean 39.7) (data not shown). Twenty (83%) of 24 LAMP-negative samples had a C_t value of 40 (i.e. no detectable amplification). The IS*481* real-time PCR and LAMP results showed a high level of agreement (77/83; 93%) with 55/83 found to be positive in both assays and 22/83 found to be negative in both assays, when a C_t value >37.6 was used as the cut-off for the real-time PCR.

B. pertussis DNA loads among children and adults

Figure 2a shows C_t values of LAMP-positive DNA samples from 40 children (mean age 6.0 years; age range 0–15 years) and 19 adults (mean age 43.3 years; age range 22–83 years). The child samples had C_t values in the range 14.6–39.5 (mean

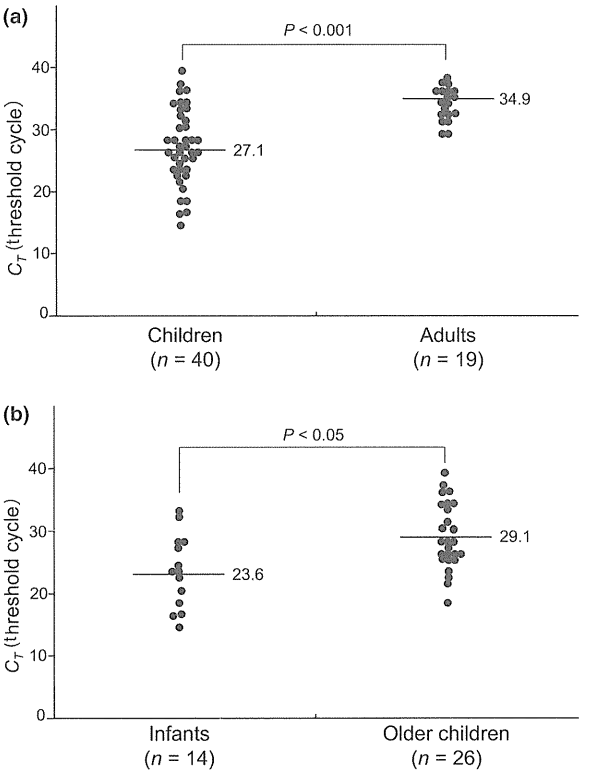

FIG. 2. Comparison of *Bordetella pertussis* DNA loads in nasopharyngeal swabs among children and adults with pertussis. (a) Children (infant and older children, $n = 40$) versus adults ($n = 19$). (b) Infants ($n = 14$) versus older children ($n = 26$). The DNA samples (2 μL) were subjected to IS*481* real-time PCR. Horizontal bars indicate mean C_t values.

27.1), whereas the adult samples had significantly higher C_t values in the range 29.1–39.0 (mean 34.9) within a narrow range. Statistical significance was observed with respect to the C_t values between children and adults ($p < 0.001$). Figure 2b also shows C_t values of LAMP-positive DNA samples from 14 infants (mean age 2.9 months; age range 1–6 months) and 26 older children (mean age 9.2 years; age range 2–15 years). The infant samples had C_t values in the range 14.6–33.8 (mean 23.6). By contrast, the older child samples had higher C_t values in the range 18.8–39.5 (mean 29.1) (p 0.011).

The clinical information about cough duration was obtained from 16 adult patients. Eight adults had a cough duration of less than 14 days (mean 8.6 days) at the time of sampling, and other adults had a duration of more than 15 days (mean 30.3 days). The C_t values (mean C_t value of ≤14 days, 35.9; >15 days, 34.8) were not statistically significant between these two groups (p 0.92) (data not shown).

Relationship of *B. pertussis* DNA loads and bacterial genotypes

On the basis of the MLST analysis, Japanese *B. pertussis* isolates could be classified into five genotypes (MLST-1 to MLST-5), as described previously [2]. Among 40 children NPS samples, the MLSTs in 33 samples were identified as: 17 MLST-1 (harboring *ptxA2*, *prn1* and *fim3A* alleles); 14 MLST-2 (*ptxA1*, *prn2* and *fim3A*); and two MLST-4 (*ptxA1*, *prn2* and *fim3B*). The mean C_t values for MLST-1, -2, and -4 were 27.0, 24.8, and 28.6, respectively. Comparison of C_t values for MLST-1 and -2 revealed no statistically significant difference (p 0.27) (data not shown). Among the infants and older children, no correlation was found between *B. pertussis* DNA loads and the bacterial genotypes.

Discussion

To our knowledge, this is the first report of a precise comparative analysis of *B. pertussis* loads in adults and children. The results obtained clearly indicate that adults had very low *B. pertussis* DNA loads in their NPS compared to children, especially infants. When bacterial loads in NPS were calculated for *B. pertussis* Tohama cells using a standard curve, the mean numbers of bacterial cells taken with a rayon-tipped swab from adults, older children and infants were estimated to be 320 (95% CI 120–910), 2.1×10^4 (95% CI 5.3×10^3 to 8.3×10^4) and 1.1×10^6 cells (95% CI 1.2×10^5 to 8.9×10^6), respectively (Table 2). Surprisingly, the bacterial numbers in adults were 340-fold and 65-fold lower than those in infants and older children, respectively. In general, adults with pertussis showed only prolonged cough illness and had less typical symptoms than children [3,6–9]. In addition, vaccinated asymptomatic children had significantly fewer *B. pertussis* than symptomatic patients [21]. Our experimental observations strongly suggest that the lower nasopharyngeal bacterial load in adults is related to the atypical and milder symptoms in adult pertussis.

TABLE 2. Number of *Bordetella pertussis* cells taken with a rayon-tipped swab from infant, child and adult patient

Patient	Number of patients	Mean age (range)	*B. pertussis* cells/swab (95% CI)[a]
Infant	14	2.9 months (1–6 months)	1.1×10^6 (1.2×10^5 to 8.9×10^6)
Older child	26	9.2 years (2–15 years)	2.1×10^4 (5.3×10^3 to 8.3×10^4)
Adult	19	43.3 years (22–83 years)	320 (120–910)

[a]Bacterial cells were calculated for *B. pertussis* Tohama cell.

Previously, Bidet *et al.* [16] demonstrated that *B. pertussis* DNA loads in NPS decreased progressively during antibiotic treatment in children. Several factors are also considered to affect *B. pertussis* load in the human nasopharynx, including patient age, previous vaccination or infection, and host genetic background. In the present study, infants had a larger *B. pertussis* DNA load than older children, and most (85%) of the infants had not received pertussis vaccination, whereas most (83%) of the older children had received four doses of the vaccine. Our findings support the hypothesis that the *B. pertussis* DNA load is affected by vaccination. Unfortunately, this does not apply to adults because pertussis vaccines are unable to provide lifelong immunity [22]. The duration of immunity post-vaccination is estimated to be in the range 4–12 years and, therefore, the bacterial loads in adults may be affected by other factor(s) besides the vaccination.

B. pertussis culture has been taken as a gold standard diagnostic method because it is highly specific. However, the culture has limited sensitivity for previously vaccinated persons, especially adolescents and adults [5,8,9]. In the present study, we demonstrated that adults with pertussis had very low *B. pertussis* DNA loads in their NPS during both early and later stages of the cough illness. This finding suggests that the diagnosis of pertussis by bacterial culture is difficult in adults even if NPS are obtained in the early stage of the illness. Compared with the culture method, nucleic acid amplification tests such as IS*481*-based real-time PCR and *B. pertussis*-specific LAMP provide a highly sensitive procedure for detection of *B. pertussis* DNA [11,13,14]. On the basis of our findings, we recommend the performance of

nucleic acid amplification tests for an accurate diagnosis of pertussis, especially when adult pertussis cases are suspected.

During the last three decades, genetic divergence in *B. pertussis* has been observed in many countries [23–27]. In Japan, the circulating strains have shifted mainly from MLST-1 to MLST-2. The MLST-1 strains include vaccine-types *ptxA2* and *prn1* alleles, whereas the MLST-2 strains include nonvaccine-types *ptxA1* and *prn2* alleles [2]. This genetic shift has been speculated to have resulted from adaptation of the bacterial population to vaccine-induced immunity [23,24,28,29]. The bacterial load of the emerging MLST-2 strains in the nasopharynx has not previously been investigated. We therefore examined the bacterial DNA loads of MLST-2 and MLST-1 strains but found no association between the DNA loads and their genotypes among infants and older children. This would suggest that emerging MLST-2 strains have the ability to produce similar bacterial loads to those of the the MLST-1 strains.

In conclusion, the *B. pertussis* DNA load in NPS depends highly on patient age. Adults had very low *B. pertussis* DNA loads and organism numbers in their NPS during both the early and later stages of the cough illness, and this explains the culture-negative results in adult pertussis cases. To make an accurate diagnosis of adult pertussis, nucleic amplification tests such as the IS*481* real-time PCR and *B. pertussis*-specific LAMP assays are recommended as sensitive methods.

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Transparency Declaration

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References

1. Mattoo S, Cherry JD. Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other *Bordetella* subspecies. *Clin Microbiol Rev* 2005; 18: 326–382.
2. Han HJ, Kamachi K, Okada K *et al.* Antigenic variation in *Bordetella pertussis* isolates recovered from adults and children in Japan. *Vaccine* 2008; 26: 1530–1534.
3. Birkebaek NH, Kristiansen M, Seefeldt T *et al. Bordetella pertussis* and chronic cough in adults. *Clin Infect Dis* 1999; 29: 1239–1242.
4. Hewlett EL, Edwards KM. Pertussis – not just for kids. *N Engl J Med* 2005; 352: 1215–1222.
5. von König CH, Halperin S, Riffelmann M, Guiso N. Pertussis of adults and infants. *Lancet Infect Dis* 2002; 2: 744–750.
6. Cherry JD, Grimprel E, Guiso N, Heininger U, Mertsola J. Defining pertussis epidemiology clinical, microbiology and serologic perspectives. *Pediatr Infect Dis J* 2005; 24: 25–34.
7. Crowcroft NS, Pebody RG. Recent developments in pertussis. *Lancet* 2006; 367: 1926–1936.
8. Greenberg DP. Pertussis in adolescents: increasing incidence brings attention to the need for booster immunization of adolescents. *Pediatr Infect Dis J* 2005; 24: 721–728.
9. Wright SW, Edwards KM, Decker MD, Zeldin MH. Pertussis infection in adults with persistent cough. *JAMA* 1995; 273: 1044–1046.
10. Guthrie JL, Seah C, Brown S, Tang P, Jamieson F, Drews SJ. Use of *Bordetella pertussis* BP3385 to establish a cutoff value for an IS*481*-targeted real-time PCR assay. *J Clin Microbiol* 2008; 46: 3798–3799.
11. Kamachi K, Toyoizumi-Ajisaka H, Toda K *et al.* Development and evaluation of loop-mediated isothermal amplification method for rapid diagnosis of *Bordetella pertussis* infection. *J Clin Microbiol* 2006; 44: 1899–1902.
12. Kösters K, Riffelmann M, von König CH. Evaluation of a real-time PCR assay for detection of *Bordetella pertussis* and *B. parapertussis* in clinical samples. *J Med Microbiol* 2001; 50: 436–440.
13. Riffelmann M, von König CH, Caro V, Guiso N. Nucleic acid amplification tests for diagnosis of *Bordetella* infections. *J Clin Micribiol* 2005; 43: 4925–4929.
14. Dragsted DM, Dohn B, Madsen J, Jensen JS. Comparison of culture and PCR for detection of *Bordetella pertussis* and *Bordetella parapertussis* under routine laboratory conditions. *J Med Microbiol* 2004; 53: 749–754.
15. Fry NK, Duncan J, Wanger K *et al.* Role of PCR in the diagnosis of pertussis infection in infants: 5 years' experience of provision of a same-day real-time PCR service in England and Wales from 2002 to 2007. *J Med Microbiol* 2009; 58: 1023–1029.
16. Bidet P, Liguori S, De Lauzanne A *et al.* Real-rime PCR measurement of persistence of *Bordetella pertussis* DNA in nasopharyngeal secretions during antibiotic treatment of young children with pertussis. *J Clin Microbiol* 2008; 46: 3636–3638.
17. Bonacorsi S, Farnoux C, Bidet P *et al.* Treatment failure of nosocomial pertussis infection in a very-low-birth-weight neonate. *J Clin Microbiol* 2006; 44: 3830–3832.
18. Greiner O, Day PJ, Bosshard PP, Imeri F, Altwegg M, Nadal D. Quantitative detection of *Streptococcus pneumoniae* in nasopharyngeal secretions by real-time PCR. *J Clin Microbiol* 2001; 39: 3129–3134.
19. Greiner O, Day PJ, Altwegg M, Nadal D. Quantitative detection of *Moraxella catarrhalis* in nasopharyngeal secretions by real-time PCR. *J Clin Microbiol* 2003; 41: 1386–1390.
20. Parkhill J, Sebaihia M, Preston A *et al.* Comparative analysis of the genome sequences of *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. *Nat Genet* 2003; 35: 32–40.
21. He Q, Arvilommi H, Viljanen MK, Mertsola J. Outcomes of *Bordetella* infections in vaccinated children: effects of bacterial number in the nasopharynx and patient age. *Clin Diagn Lab Immunol* 1999; 6: 534–536.
22. Wendelboe AM, Van Rie A, Salmaso S, Englund JA. Duration of immunity against pertussis after natural infection or vaccination. *Pediatr Infect Dis J* 2005; 24: 58–61.
23. Borisova O, Kombarova SY, Zakharova NS *et al.* Antigenic divergence between *Bordetella pertussis* clinical isolates from the Moscow, Russia, and vaccine strains. *Clin Vaccine Immunol* 2007; 14: 234–238.

24. Cassiday P, Sanden G, Heuvelman K, Mooi F, Bisgard KM, Popovic T. Polymorphism in *Bordetella pertussis* pertactin and pertussis toxin virulence factors in the United States, 1935–1999. *J Infect Dis* 2000; 182: 1402–1408.
25. Kodama A, Kamachi K, Horiuchi Y, Konda T, Arakawa Y. Antigenic divergence suggested by correlation between antigenic variation and pulsed-field gel electrophoresis profiles of *Bordetella pertussis* isolates in Japan. *J Clin Microbiol* 2004; 42: 5453–5457.
26. Litt DJ, Neal SE, Fry NK. Changes in genetic diversity of the *Bordetella pertussis* population in the United Kingdom between 1920 and 2006 reflect vaccination coverage and emergence of a single dominant clonal type. *J Clin Microbiol* 2009; 47: 680–688.
27. Tsang RS, Lau AK, Sill ML *et al.* Polymorphisms of the fimbria *fim3* gene of *Bordetella pertussis* strains isolated in Canada. *J Clin Microbiol* 2004; 42: 5364–5367.
28. He Q, Mäkinen J, Berbers G *et al. Bordetella pertussis* protein pertactin induces type-specific antibodies: one possible explanation for the emergence of antigenic variants? *J Infect Dis* 2003; 187: 1200–1205.
29. Mooi FR, van Oirschot H, Heuvelman K, van der Heide HG, Gaastra W, Willems RJ. Polymorphism in the *Bordetella pertussis* virulence factors P.69/pertactin and pertussis toxin in the Netherlands: temporal trends and evidence for vaccine-driven evolution. *Infect Immun* 1998; 66: 670–675.

Differential Expression of Type III Effector BteA Protein Due to IS*481* Insertion in *Bordetella pertussis*

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Abstract

Background: *Bordetella pertussis* is the primary etiologic agent of the disease pertussis. Universal immunization programs have contributed to a significant reduction in morbidity and mortality of pertussis; however, incidence of the disease, especially in adolescents and adults, has increased in several countries despite high vaccination coverage. During the last three decades, strains of *Bordetella pertussis* in circulation have shifted from the vaccine-type to the nonvaccine-type in many countries. A comparative proteomic analysis of the strains was performed to identify protein(s) involved in the type shift.

Methodology/Principal Finding: Proteomic analysis identified one differentially expressed protein in the B. pertussis strains: the type III cytotoxic effector protein BteA, which is responsible for host cell death in Bordetella bronchiseptica infections. Immunoblot analysis confirmed the prominent expression of BteA protein in the nonvaccine-type strains but not in the vaccine-type strains. Sequence analysis of the vaccine-type strains revealed an IS481 insertion in the 5′ untranslated region of bteA, −136 bp upstream of the bteA start codon. A high level of bteA transcripts from the IS481 promoter was detected in the vaccine-type strains, indicating that the transcript might be an untranslatable form. Furthermore, BteA mutant studies demonstrated that BteA expression in the vaccine-type strains is down-regulated by the IS481 insertion.

Conclusion/Significance: The cytotoxic effector BteA protein is expressed at higher levels in B. pertussis nonvaccine-type strains than in vaccine-type strains. This type-dependent expression is due to an insertion of IS481 in B. pertussis clinical strains, suggesting that augmented expression of BteA protein might play a key role in the type shift of B. pertussis.

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Introduction

Bordetella pertussis is a human-specific pathogen that is the etiologic agent of whooping cough, an acute respiratory disease that is often particularly severe in infants [1]. Universal immunization programs have contributed to a significant reduction in morbidity and mortality of pertussis, especially in infants and children; however, the incidence of pertussis has increased in several countries despite high vaccination coverage [2–5]. Since the 1980s, a considerable genetic transition has been observed between *B. pertussis* vaccine strains and circulating clinical strains in many countries [6–11]. Genetic variations have been found in the loci encoding the major *B. pertussis* virulence factors: pertussis toxin S1 subunit (*ptxA*), pertactin (*prn*) and fimbriae 3 (*fim3*). Among circulating *B. pertussis* strains, vaccine-type alleles (*ptxA2*, *prn1* and *fim3A*) have been replaced mainly with nonvaccine-type alleles (*ptxA1*, *prn2* and *fim3B*). It has been speculated that adaptation of the bacterial population to vaccine-induced immunity has produced this genetic shift, and is one possible explanation for the resurgence of pertussis [12–15]. However, there have been few reports of the exact mechanism underlying this phenomenon.

B. pertussis expresses various virulence factors, including adhesins and toxins, which function to establish and maintain host infection. Several virulence factors such as filamentous haemagglutinin (FHA) and pertussis toxin (PT) are expressed under the control of the BvgAS two-component regulatory system [1,16,17]. The BvgAS system also positively regulates virulence factor secretion via the type III secretion system (T3SS) [18,19]. T3SS is highly conserved among a number of Gram-negative bacteria and functions as an injector of virulence molecules (i.e., effectors) into the host cell through a needle-like injection apparatus [20,21]. In *B. pertussis*, T3SS plays a role in subverting the protective innate and adaptive immunity of the host. Three T3SS-secreted proteins, BopN, BopD and Bsp22, have been identified so far [22]. In the animal pathogen *Bordetella bronchiseptica*, BopN is involved in the up-regulation of cytokine IL-10 [23], while Bsp22 polymerizes to form a flexible filamentous structure at the tip of the needle structure and associates with the pore component BopD [24]. The Bsp22 translocon is expressed in a significant proportion of *B. pertussis* clinical isolates but not in Tohama and Wellcome 28, the common laboratory-adapted vaccine strains [22].

Genomic differences between *B. pertussis* clinical strains and the vaccine strain Tohama have been investigated. The comparative

genomics profiling revealed that the genome of *B. pertussis* Tohama differs from clinical isolates in four regions (RD11 to RD14) [25]. In contrast, progressive gene loss mediated by homologous recombination between IS*481* insertion sequence elements has been observed among recently circulating strains of *B. pertussis* isolates [26,27]. IS*481* is present in multiple copies on the *B. pertussis* chromosome, and it plays a critical role in *B. pertussis* evolution through genomic rearrangement.

Proteomic analysis has been widely applied to comparisons of protein expression among different strains, and information accumulated from genomic studies of *Bordetella* spp. facilitates comparative proteomic approaches to the investigation of *B. pertussis* clinical strains [6,28]. In the present study, a proteomic approach was employed to identify the protein(s) involved in the genetic shift from vaccine-type to nonvaccine-type in *B. pertussis* strains. The protein profile analyses identified one differentially expressed protein, the T3SS effector BteA (alias BopC) [29,30], between the strain types. BteA is a 68 kDa cytotoxic effector that has been identified in *B. bronchiseptica* but not in the *B. pertussis* human pathogen. Here we studied the differential expression of BteA protein in *B. pertussis* clinical strains and identified a specific IS*481* insertion in the 5′ untranslated region (5′-UTR) of *bteA* in vaccine-type strains.

Results

Identification of BteA in *B. pertussis* nonvaccine-type strain

A comparative proteomic analysis of two clinical strains was performed to investigate the shift of *B. pertussis* strains from vaccine-type to nonvaccine-type. Figure 1 shows 2-dimensional electrophoretic (2-DE) maps of total protein expressed in the nonvaccine-type clinical strain BP235 and the vaccine-type BP233. Among >600 protein spots detected on the 2-DE gel, one was notably absent in the 2-DE map of BP233. The protein spot was observed in other nonvaccine-type strains (BP157, BP159, BP162 and BP228), but not in other vaccine-type strains (BP155, BP156, BP232 and BP243). The protein represented by the spot was identified by LC-MS/MS analysis using tryptic digests. The MS/MS of the protein digests provided four peptide sequences (RPDEFAAR, FDALR, ITALNLR and TQTQLLALQR) that matched the *B. pertussis* hypothetical protein BP0500 (NCBI accession: NP_879352). Hypothetical protein BP0500 was identified as the T3SS effector BteA, since the sequence is highly conserved with 98% amino acid identity to the BteA (BopC) of *B. bronchiseptica* [29,30].

High expression of BteA protein in nonvaccine-type strains

Immunoblots of *B. pertussis* clinical strains using anti-BteA antiserum detected high levels of a protein of ~68 kDa in all nonvaccine-type clinical strains (BP157, BP159, BP162, BP228 and BP235), whereas BteA expression was greatly reduced in the vaccine-type clinical strains (BP155, BP156, BP232, BP233 and BP243). Additional products of >200 kDa were also detected in the nonvaccine-type clinical strains. These high molecular mass signals appear to be the protein bands that have been reported as a multimeric complex of BteA in *B. bronchiseptica* [29,30] (see Figure S1). T3SS function in the nonvaccine-type strains was confirmed

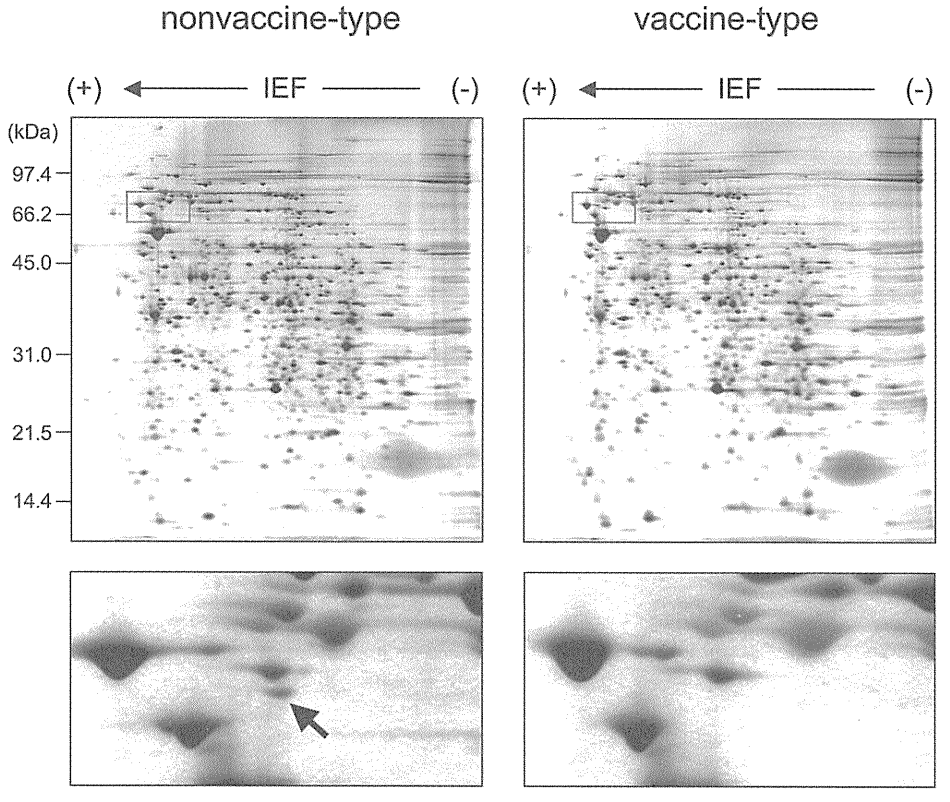

Figure 1. Comparative proteomic analysis of *B. pertussis* nonvaccine-type and vaccine-type strains. Total protein (10 μg) from the nonvaccine-type and vaccine-type clinical strains was separated by 2-D gel electrophoresis and silver stained. The left upper panel shows the protein profile of the nonvaccine-type BP235. The right upper panel shows the protein profile of the vaccine-type BP233. The red-boxed areas are enlarged (lower panels). The arrow in left lower panel indicates the spot that was identified as type III effector BteA by LC-MS/MS analysis.
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by using whole cell protein extracts for immunoblots of BtcA (the BteA chaperone) [29,31] and BopD (the T3SS translocon) [32]. BtcA and BopD polypeptides were detected in both strain types, but the BtcA signals produced by the nonvaccine-type strains were apparently lower than those of the vaccine-type strains (Figure 2). The reason for the different expression is not clear. In contrast, adenylate cyclase toxin (ACT), another *Bordetella* spp. virulence factor, was detected at similar levels in both strain types.

In order to confirm BteA secretion by *B. pertussis* strains, BteA polypeptide in the culture supernatants (CS) was subjected to immunoblot analysis. BteA was detected in secreted proteins from the nonvaccine-type clinical strain BP159 at 12, 24 and 48 h, whereas the signal was very low in the vaccine-type clinical strain BP155 over the 48-h time period (Figure 3). Conversely, signals corresponding to PT-S1 subunit and FHA polypeptides were detected in the supernatants of both cultures throughout the sampling period, although silver staining revealed small differences in their protein profiles after 24 h in culture.

Transcription of *bteA*

bteA gene expression in *B. pertussis* strains was investigated with conventional RT-PCR and quantitative RT-PCR. *bteA* was transcribed in both the nonvaccine-type (BP157, BP159, BP162, BP228 and BP235) and vaccine-type (BP155, BP156, BP232, BP233 and BP243) clinical strains (Figure 4A). Similarly, *bteA* transcripts were detected in both strain groups. RT-PCR experiments lacking reverse transcriptase showed no specific product for *bteA* amplification, confirming negligible genomic DNA contamination in the RNA preparations. Quantitative RT-PCR (qRT-PCR) showed an average *bteA* transcript level of 0.146 (±1SD range, 0.107 to 0.184) in nonvaccine-type strains and 0.095 (±1SD range, 0.076 to 0.113) in vaccine-type clinical strains, a difference that was not statistically significant ($P=0.11$) (Figure 4B).

IS*481* insertion in the *bteA* 5′-UTR in vaccine-type strains

Sequencing of the *bteA* 5′-UTR of the five vaccine-type strains (BP155, BP156, BP232, BP233 and BP243), revealed a 1,043-bp insertion sequence (IS*481*) −136 bp upstream of the *bteA* start codon (Figure 5A). IS*481a*, which is newly identified in *B. pertussis*, showed 99% nucleotide sequence identity with IS*481* of *B. pertussis* Tohama. The CCTAAC sequence in the *bteA* 5′-UTR is an insertion site of IS*481a* and is duplicated by the insertion, although the 6-bp consensus recognition sequence of IS*481* has been reported as NCTAGN [33]. IS*481* insertions were not found in the nonvaccine-type clinical strains, which had nucleotide sequences that were 99% identical to that of *B. pertussis* Tohama. In the *bteA* 5′-UTR of the nonvaccine-type strains (BP157, BP159, BP162 BP228 and BP235), one single nucleotide polymorphism (A→G) was observed at 207 bp upstream of the *bteA* translation start site (Figure 5B).

The *bteA* 5′-UTR was PCR-amplified from chromosomal DNA of other *B. pertussis* strains to confirm insertion of IS*481*. Among 61 vaccine-type clinical strains, 60 (98%) produced amplicons of ~3.1 kb, a size indicative of an IS*481* insertion in the *bteA* 5′-UTR. One strain (BP121) had a product of ~2.1 kb, corresponding to the predicted size of the native 5′-UTR (data not shown). Of the 23 nonvaccine-type strains examined, all generated ~2.1 kb amplicons, confirming the absence of the IS*481* insertion.

Determination of the *bteA* transcription start site

5′-RACE mapping was used to identify the *bteA* transcription start site in vaccine-type strain BP155. Nucleotide sequences of the 5′-RACE PCR products revealed two transcription start sites, P1 and P2, located −68 and −147 bp from the *bteA* translation start codon (Figure 5A). The P1 start site (+1) was located within the *bteA* 5′-UTR, whereas the P2 start site (−79) was located within IS*481a*. Only the P1 start site was also found in the nonvaccine-type strain BP159 (Figure 5B). IS*481* contains an outward-facing

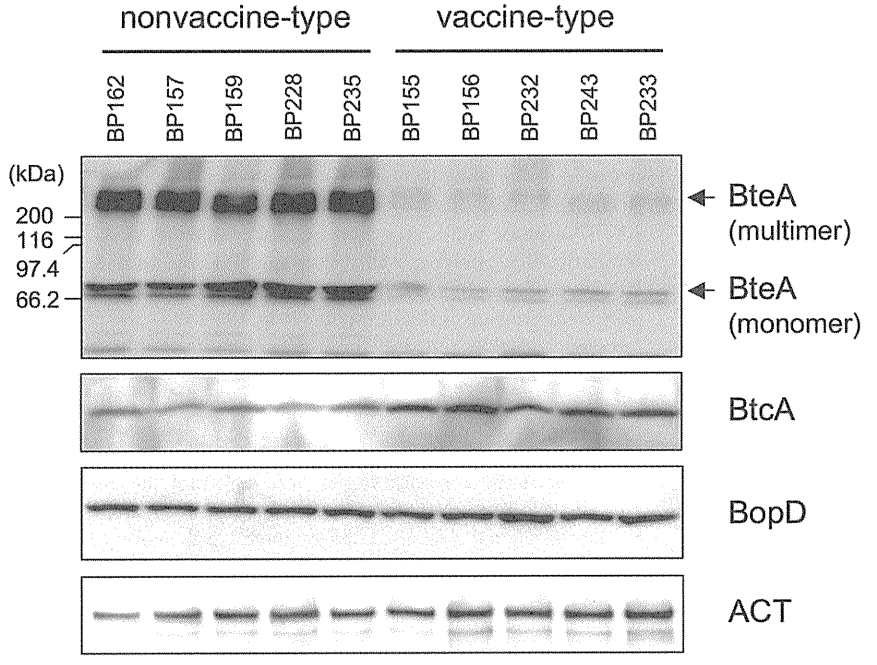

Figure 2. Expression of BteA, BtcA, BopD and ACT proteins in *B. pertussis* nonvaccine-type and vaccine-type strains. The nonvaccine-type clinical strains (BP157, BP159, BP162, BP228 and BP235) and vaccine-type clinical strains (BP155, BP156, BP232, BP233 and BP243) were cultured in modified SS medium for 18 h. Total protein extracted from bacterial cells was subjected to immunoblot analysis with anti-BteA, anti-BtcA, anti-BopD or anti-ACT antiserum. For BteA detection, 10 μg of total protein was loaded in each lane.
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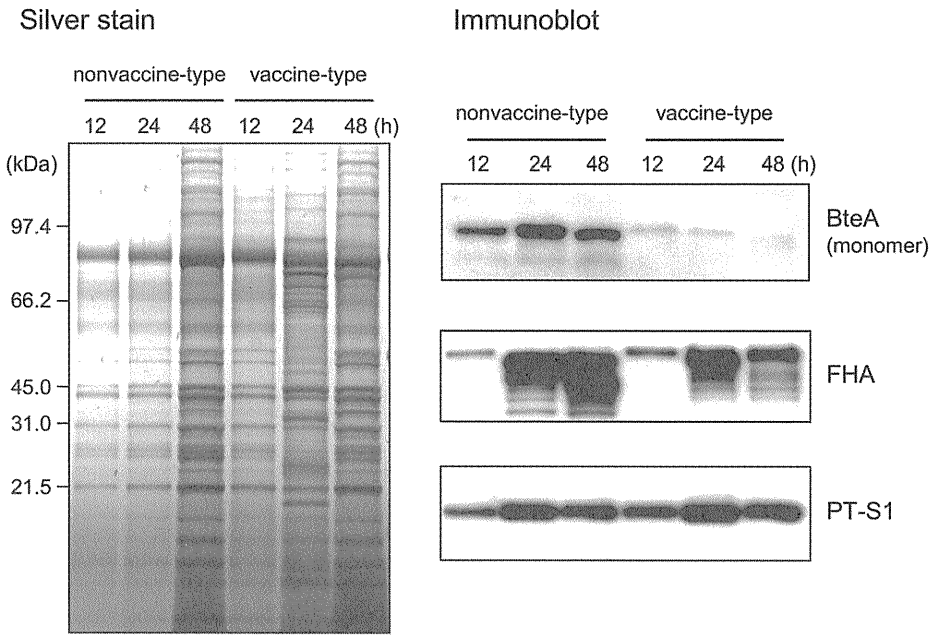

Figure 3. BteA secretion from *B. pertussis* nonvaccine-type and vaccine type strains. Strains BP235 (nonvaccine-type) and BP233 (vaccine-type) were cultured in modified SS medium, and the culture supernatants (CS) were collected at 12, 24 and 48 h. Protein samples prepared by precipitation with 10% trichloroacetic acid were separated by 12.5% SDS-PAGE followed by silver staining (left panel). BteA, FHA and PT secretions were analyzed by immunoblots using anti-BteA, anti-FHA or anti-PT antiserum (right panels). For BteA detection, the equivalent of 200 μl of CS was loaded in each lane.
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promoter at one end that is responsible for transcription of the flanking catalase gene (*katA*) in *B. pertussis* [34]. However, the P2 start site is different from the *katA* transcription start site. The transcription start site of *btcA*, also determined by 5′-RACE, was mapped to a T residue 31 bp upstream of the *btcA* translation start codon in both the vaccine-type and nonvaccine-type strains (Figure 5B).

Primer extension analysis was also performed in an attempt to resolve the *bteA* transcription start sites. However, the start sites could not be ascertained, probably due to low amounts of *bteA* transcript in *B. pertussis*.

IS*481a*-promoter transcript is the major *bteA* transcript in the vaccine-type strain

Expression of the IS*481a*-promoter transcript (P2 transcript) in *B. pertussis* vaccine-type strain BP155 was analyzed by qRT-PCR with TaqMan probes (Figure 5A). The P2 transcript and total *bteA* (P1 + P2) transcripts were determined individually and the ratio of P2 transcript to total *bteA* transcript was calculated. Based on four independent experiments, the ratio (P2 transcript/P1 + P2 transcripts) was estimated to be 0.88 (±1SD range, 0.70 to 1.09), indicating that the P2 transcript is the major *bteA* transcript in the vaccine-type strain (data not shown).

BteA expression in *B. pertussis* BteA mutants

To clarify the effect of the IS*481* insertion on BteA expression, four BteA mutants (Δ*bteA*-BP155, ΔIS*481*-BP155, Δ*bteA*-BP157 and +IS*481*-BP157) were constructed from *B. pertussis* BP155 (vaccine-type) and BP157 (nonvaccine-type) by homologous recombination (Figure 6A). The Δ*bteA*-BP155 and Δ*bteA*-BP157 mutants had a 178-bp deletion in the 5′ region of *bteA*. In the ΔIS*481*-BP155 mutant, a 2.2-kb insertion containing an intact *bteA* 5′-UTR (derived from *B. pertussis* Tohama) replaced the native *bteA* 5′-UTR + IS*481a* gene. In contrast, +IS*481*-BP157 mutant had a 3.2-kb insertion containing a *bteA* 5′-UTR + IS*481a* (derived from *B. pertussis* BP155) instead of its own *bteA* 5′-UTR. Consequently, ΔIS*481*-BP155 had an IS*481a* deletion from the *bteA* 5′-UTR, whereas the +IS*481*-BP157 mutant had an IS*481a* insertion in the *bteA* 5′-UTR. The *btcA*−*bteA* region of the mutants was verified by DNA sequence analysis.

BteA expression in the bacterial cells and CS after 24 h in culture was analyzed by immunoblot with anti-BteA antiserum (Figure 6B). In ΔIS*481*-BP155 bacterial cells and CS, BteA polypeptide(s) corresponding to ~68 kDa and >200 kDa were detected at the same level as was observed in the BP157 wild-type strain. In contrast, the signals of BteA polypeptide(s) from +IS*481*-BP157 mutant were very low in both bacterial cells and CS. Similarly, BteA polypeptide(s) were not detected in either Δ*bteA*-BP155 or Δ*bteA*-BP157. These results clearly showed that BteA protein expression is down-regulated by the IS*481* insertion in *B. pertussis*, and that the anti-BteA antiserum is highly specific to BteA.

Discussion

The BteA effector (alias BopC) is required for the induction of necrotic cell death during *B. bronchiseptica* infections, and is thought to play a pivotal role in T3SS-mediated cell death [29,30,35]. BteA is also involved in dephosphorylation of tyrosine-phosphorylated proteins (PY) of host cells [30], and its 130-amino acid N-terminal sequence is associated with target lipid rafts [31]. BteA is the only cytotoxic effector that has been identified in *Bordetella* spp. In *B. pertussis*, low-passage clinical strains have an ability to express a functionally active T3SS; however, BteA protein had not been detected in the clinical and common laboratory-adapted strains by MALDI-TOF mass spectrometry [22]. Here we demonstrate that BteA protein is highly expressed in *B. pertussis* nonvaccine-type

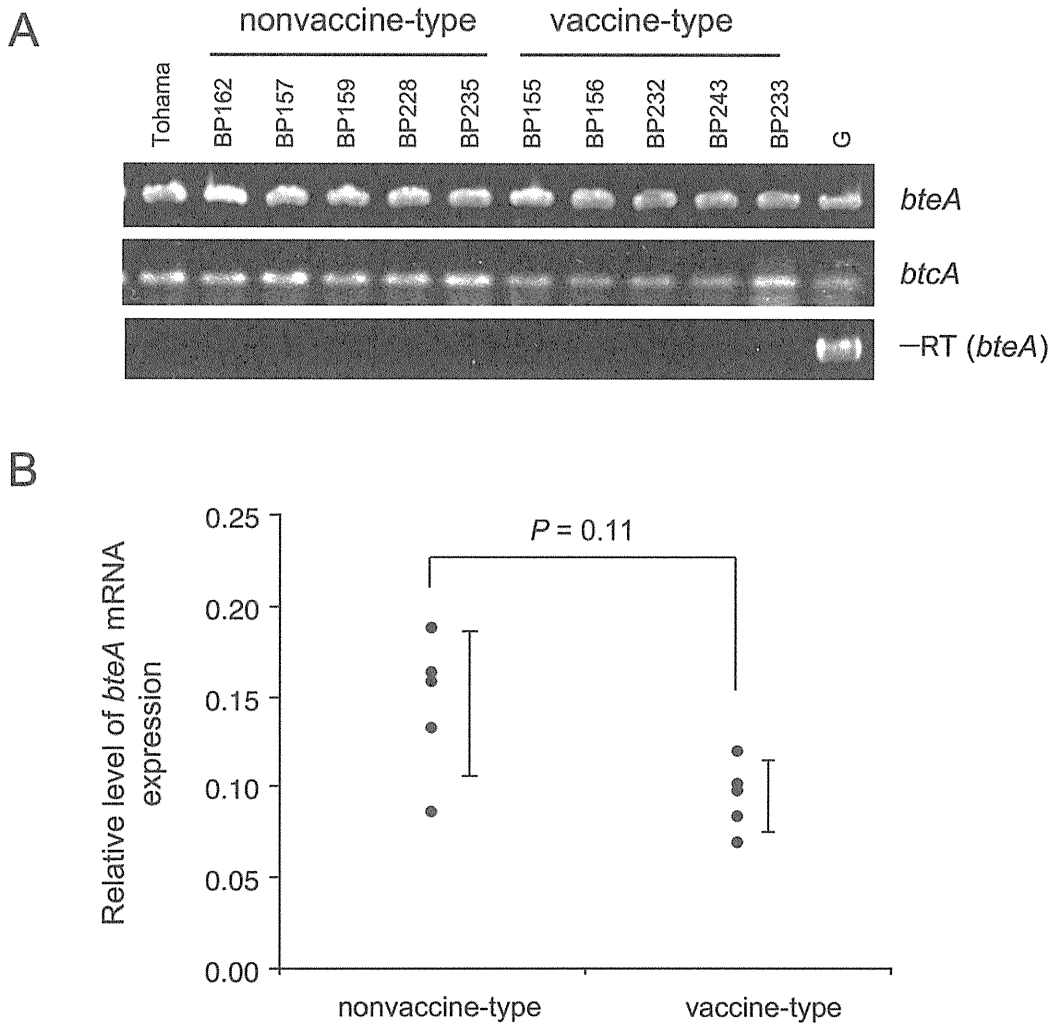

Figure 4. RT-PCR analysis of *bteA* transcript in *B. pertussis* nonvaccine-type and vaccine-type strains. (A) RT-PCR with primers specific for *bteA* and *btcA*. cDNA made from total RNA of nonvaccine-type (BP157, BP159, BP162, BP228 and BP235) and vaccine-type (BP155, BP156, BP232, BP233 and BP243) clinical strains was used as templates for PCR. Genomic DNA (G) from *B. pertussis* strain Tohama was used as a positive control. A mock reaction for *bteA* (−RT) consisted of reactions lacking reverse transcriptase. (B) Quantitative RT-PCR analysis of *bteA* transcript levels in the nonvaccine-type and vaccine-type clinical strains listed in (A). The *recA* transcript was used as a reference. Each point represents one strain and vertical bars indicate standard deviations.
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strains but not in the vaccine-type strains, and that BteA protein expression is down-regulated by IS*481a* insertion in the vaccine-type strains. We provide the first evidence that BteA protein expression is type-dependent due to the IS*481a* insertion in *B. pertussis* clinical strains.

In Japan, *B. pertussis* circulating strains began to change from vaccine-type to nonvaccine-type in the mid-1990s [8], and the reported incidence of adult cases of pertussis has dramatically increased since 2002 [36]. The genetic divergence in *B. pertussis* circulating strains has also been observed in many other countries. A possible explanation for the genetic divergence is that the type shift is a result of vaccine-driven evolution [12–15]. More recently, Mooi et al. [37] reported that expansion of *B. pertussis* strains with increased PT production has contributed to the resurgence of pertussis in the Netherlands. Here we showed prominent expression of the T3SS effector protein BteA in the nonvaccine-type strains, and that PT and ACT (important virulence factors of *B. pertussis*) are expressed at the same level in both the nonvaccine and vaccine-type strains. Besides vaccine-driven evolution, our findings could provide another possible explanation for the type shift from vaccine-type to nonvaccine-type, i.e., the augmented expression of BteA protein in *B. pertussis* nonvaccine-type strains may be involved in the type shift.

B. bronchiseptica BteA has *in vitro* cytotoxic activity against cultured mammalian cells [18,22,29,30]. In this study, we determined the cytotoxicity of *B. pertussis* BteA mutants by measuring the release of lactate dehydrogenase (LDH) from L2 rat lung epithelial cells, J774 mouse macrophage-like cells, or HeLa cells. However, even BteA-expressing strains (Δ*IS481*-BP155 and wild-type BP157) showed low cytotoxicity (<10%), and consequently no statistically significant differences in cytotoxicity were observed among the wild-type and mutant strains. *B. pertussis* is known to have a lower *in vitro* cytotoxicity than *B. bronchiseptica* [18,22], which is consistent with the extremely low secretion of BteA in *B. pertussis* as compared to *B. bronchiseptica* (Figure S1). Therefore, a more sensitive and quantitative assay is required to determine the BteA-dependent cytotoxicity of *B. pertussis*.

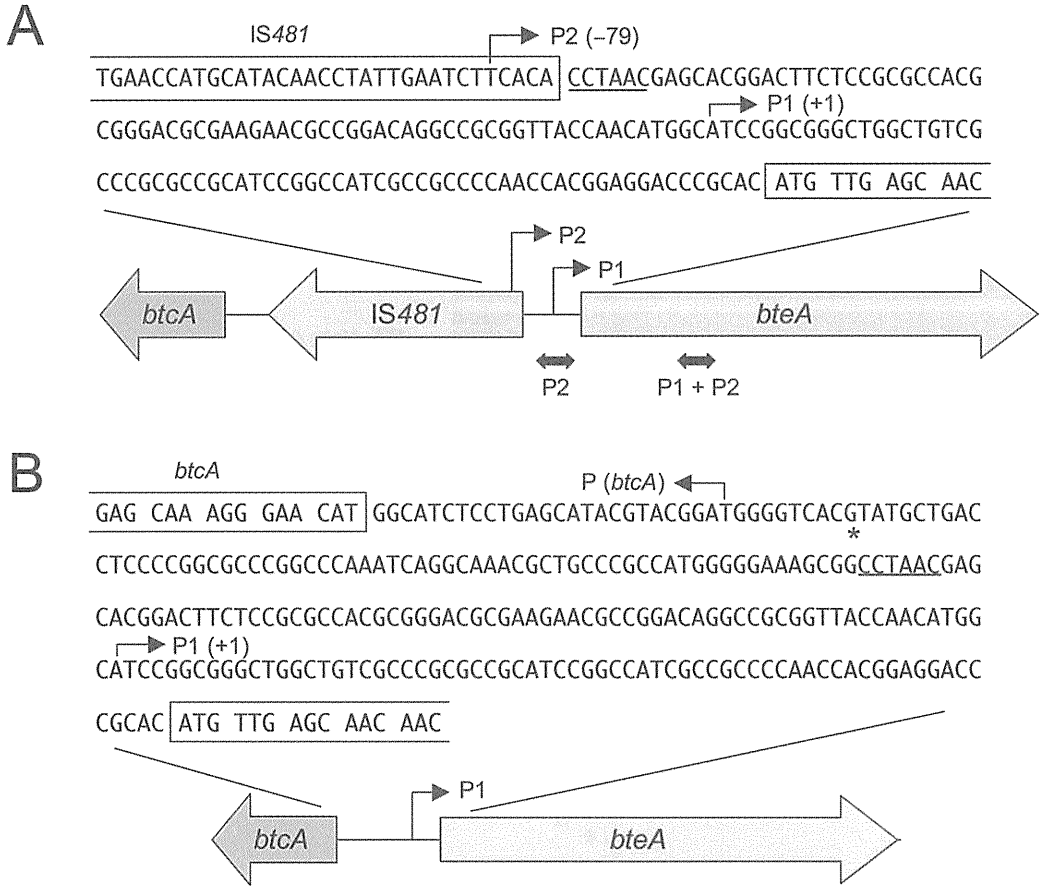

Figure 5. Physical maps of the *btcA*–*bteA* region of *B. pertussis* vaccine-type and nonvaccine-type strains. (A) The vaccine-type clinical strain BP155. The location of IS*481a* is represented by a gray arrow on the physical map. The recognition sequence of IS*481a* is underlined. The two mapped transcriptional start sites (P1 and P2) of *bteA* are shown by arrows. Region amplified by qRT-PCR to determine the IS*481a*-promoter (P2) and total (P1 + P2) transcripts are shown by two-headed arrows below the physical map. (B) The nonvaccine-type clinical strain BP159. The mapped transcriptional start sites of *bteA* (P1) and *btcA* [P (*btcA*)] are shown by arrows. The single nucleotide polymorphism (A→G) at −207 bp from the *bteA* translation start codon is indicated by an asterisk.
doi:10.1371/journal.pone.0017797.g005

IS*481* belongs to the recently defined IS*481* family [38], and 238 copies of IS*481* are present in the *B. pertussis* Tohama genome [39]. In *B. pertussis* clinical strains, IS*481* is also present in multiple copies on the chromosome and it plays a critical role in *B. pertussis* evolution [26,40]. Many IS elements have been shown to activate the expression of neighboring genes. IS*481* contains an outward-facing promoter that is located in close proximity to the left terminal inverted repeat, and this promoter is responsible for the transcription of *katA* in certain *B. pertussis* strains [34]. Here we identified an IS*481a* insertion in the *bteA* 5′-UTR in *B. pertussis* vaccine-type clinical strains and detected a high level of *bteA* transcripts from the IS*481a* promoter (P2) compared with its own promoter (P1). However, the vaccine-type strains showed a low level of BteA protein expression, suggesting that insertion of IS*481a* represses P1 promoter activity, and that P2 transcript has a low translational efficiency from the additional nucleotide sequence (79 nucleotides) at its 5′ end. Use of a cell-free coupled transcription-translation system revealed that the additional nucleotide sequence is involved in down-regulation of transcription and/or translation (Figure S2). The 5′-UTR of bacterial mRNAs can bear regulatory elements that are involved in down- or up-regulation of translation [41]. The regulatory mechanisms in this region are controlled by RNA-binding proteins, small noncoding RNAs and structural rearrangements with the 5′-UTR. In addition, a 5′ stem-loop structure that sequesters the ribosomal binding site has been shown to be involved in translational regulation. Bioinformatic analysis uncovered a predicted stem-loop structure in the *bteA* 5′-UTR of P2 transcript (Figure S2).

In this study, the 5′-UTRs of five *B. pertussis* vaccine-type clinical strains were sequenced; all had an insertion of an IS*481a* in the *bteA* 5′-UTR, both transcribed in the same direction. In one of the vaccine-type strains, BP155, the major *bteA* mRNA was transcribed from P2 in the IS*481a*-promoter. These observations raise the possibilities that (i) the P2 transcript is translated into BteA under certain environmental conditions, and (ii) the P2 transcript is translated into another novel protein by translational frame-shifting. BteA is known to be regulated by the BvgAS system and an extracytoplasmic function (ECF) sigma factor BtrS in *B. bronchiseptica* [18,29]. In *B. pertussis*, it has been suggested that expression of the T3SS translocon Bsp22 is blocked by post-transcriptional regulation [18]. However, the molecular details of the regulatory mechanism are still unclear. Further studies are needed to determine the down-regulation of BteA protein in *B. pertussis* vaccine-type clinical strains.

In conclusion, *B. pertussis* vaccine-type strains have been replaced with the nonvaccine-type strains in many countries, and the resurgence of pertussis has been observed in several

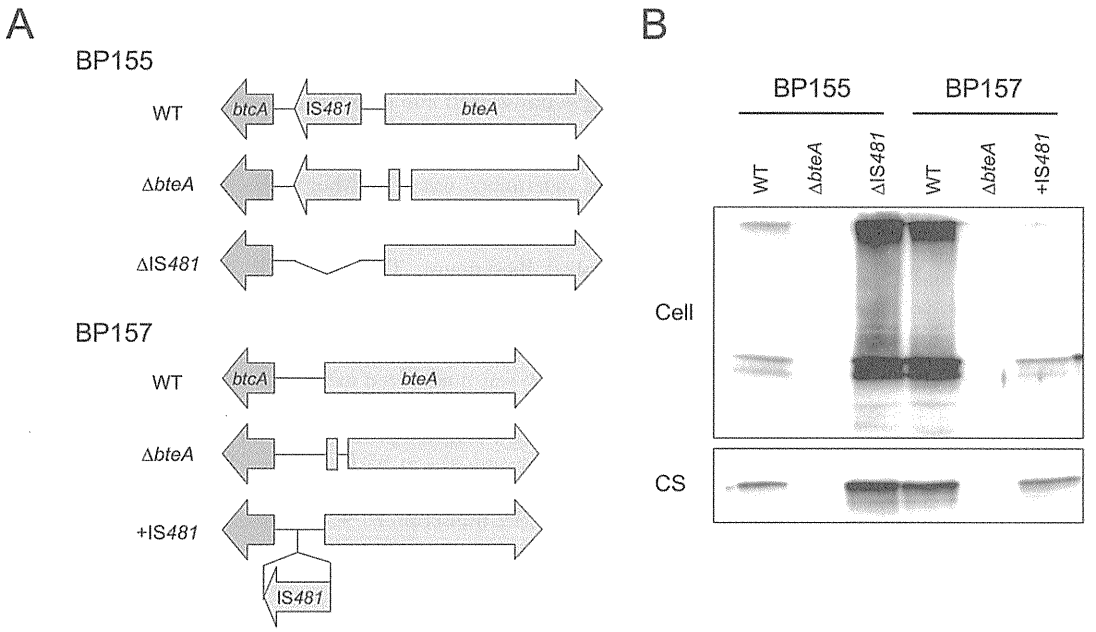

Figure 6. Construction and characterization of *B. pertussis* BteA mutants. (A) Physical map of the *btcA*–*bteA* region of BteA mutants derived from *B. pertussis* BP155 (vaccine-type) and BP157 (nonvaccine-type). WT, wild-type; Δ*bteA*, a 178-bp deletion around the 5′ region of *bteA*; Δ*IS481*, *IS481a* deletion from the *bteA* 5′-UTR; +*IS481*, *IS481a* insertion in the *bteA* 5′-UTR. (B) Expression of BteA protein in the BteA mutants. The mutants were cultured in modified SS medium for 24 h. Total protein from the bacterial cells (Cell) and culture supernatants (CS) was analyzed with immunoblot using anti-BteA antiserum.
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nations. In Japanese *B. pertussis* clinical strains, the T3SS effector BteA is highly expressed in nonvaccine-type strains as compared with the vaccine-type strains. Our findings indicate that augmented expression of BteA protein in *B. pertussis* circulating strains could play a key role in the type shift. However, it is unclear whether BteA protein is implicated in the resurgence of pertussis. Further studies are needed to determine the expression of BteA protein in *B. pertussis* circulating strains on a global scale.

Materials and Methods

Bacterial strains and growth conditions

B. pertussis clinical strains were selected from the laboratory collection of the National Institute of Infectious Diseases, Tokyo, Japan. The selection criteria included the time and geographic location of isolation, and their *ptxA* and *prn* alleles. A total of 10 clinical strains from 2002 to 2004 in Japan were included. Of the 10 clinical strains, 5 harbored *ptxA1* and *prn2* alleles (BP157, BP159, BP162, BP228 and BP235; nonvaccine-type strains), while the others carried *ptxA2* and *prn1* (BP155, BP156, BP232, BP233 and BP243; vaccine-type strains). All strains were cultured on Bordet-Gengou agar (BG agar, Difco) supplemented with 1% glycerol and 15% defibrinated horse blood or in modified Stainer-Scholte (SS) medium [42].

Two-dimensional gel electrophoresis (2D-PAGE)

2D-PAGE was performed based on the O'Farrell method [43] with minor modifications. *B. pertussis* clinical strains grown on BG agar plates were resuspended in casamino acid solution (1% casamino acid, 0.6% NaCl, pH 7.1). Bacterial cells were precipitated by centrifugation (12,000 × *g*, 10 min) and resuspended in SDS-lysis buffer (62.5 mM Tris-HCl, 1% SDS, 10% glycerol, 5% 2-mercaptoethanol, pH 6.8) by sonication. Total protein was extracted by boiling for 3 min, followed by centrifugation. A portion (10 μg, approximately 2 μl) of the protein solution was mixed with 20 μl of sample buffer [8.5 M urea, 2% Nonidet P-40, 2% Ampholine (pH 3.5 to 10)], and applied to an isoelectric focusing tube gel (2.0 mm inside diameter by 12.0 cm) containing 4% polyacrylamide, 8.5 M urea, 2% Nonidet P-40, and 2% Ampholine (pH 5 to 7 and pH 3.5 to 10 in a ratio of 1:4). Proteins were focused at 10°C for 17 h (1 h at 200 V, 2 h at 400 V, and 14 h at 800 V) with 10 mM H_3PO_4 (anolyte) and 20 mM NaOH (catholyte). In the second dimension, the electrofocused tube gel was electrophoresed in 12% SDS-PAGE. The separated polypeptides were visualized by silver staining and analyzed with the PDQuest 2-D Analysis Software (Bio-Rad, Hercules, CA). The Lowry assay was used to measure protein concentrations in a trichloroacetic acid (TCA) pellet (resuspended in 1 N NaOH) using bovine serum albumin as a standard.

Protein identification

2D-PAGE gels were stained with silver nitrate without glutaraldehyde fixation [44], and protein spots of interest were excised. Proteins were reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide, and digested with sequencing grade-modified trypsin (Promega, Madison, WI). Digested peptides were separated on a C18 capillary column (0.2 by 50 mm, Michrom Bioresources, CA) equipped with a Chorus 220 solvent delivery system and an HTC PAL auto-sampler system (CTC Analytics AG, Zwingen, Switzerland). Separated peptides were analyzed by the Finnigan LCQ^{TM} Deca XP ion trap mass spectrometer (Thermo Fisher Scientific Inc., MA) with electrospray ionization (ESI) interface using the Nanosprayer FS (GL Sciences Inc., Japan). To identify peptides, data files were generated from the MS/MS scans by Bioworks 3.0 using the SEQUEST algorithm (threshold, 10^5; minimum group scan 2, Xc >1.0, Thermo Fisher Scientific) and searched against the complete amino acid database derived from the *B. pertussis* Tohama genome database.

Antibody production against recombinant BteA, BtcA and ACT

The BteA gene (NCBI accession: NP_879352) was amplified by PCR from *B. pertussis* Tohama DNA using BteA-F and BteA-R primers, and cloned into the XmnI/HindIII sites of pMal-c2X (New England Biolabs, Beverly, MA) to generate a maltose binding protein (MBP) fusion with BteA. Production of this fusion protein was induced in *E. coli* BL21 with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and subsequently purified using amylose resin (New England Biolabs) and Resource Q (Amersham Pharmacia Biotech, Uppsala, Sweden) columns. A two-step PCR was carried out to amplify recombinant BtcA (NCBI accession: NP_879351). The first PCR was performed using the BtcA-BteA-F3 and BtcA-BteA-R3 primers (Table S1), which amplified the region between positions 165122 and 167190 of the *B. pertussis* Tohama genome (GenBank accession: BX640412). In the second PCR, *btcA* was amplified from the first PCR product with the 5-BtcA and 3-BtcA primers (Table S1) and cloned into the NdeI/HindIII sites of pCold II DNA (TAKARA Bio Inc.). His-tagged BtcA was induced in *E. coli* BL21 with 0.5 mM IPTG at 15°C and purified using the HisTrap FF Crude Kit (GE Healthcare UK Ltd.). A recombinant catalytic domain of *B. pertussis* adenylate cyclase toxin (ACT) was a gift from Minoo Watanabe.

Antibodies against MBP-BteA, BtcA and ACT were generated in mice at Nippon Biotest Laboratories, Inc. (Tokyo, Japan). The MBP-BteA antiserum was pre-absorbed with MBP2 protein (New England BioLabs) and the resulting antiserum was used.

Immunoblot analysis

B. pertussis clinical strains were inoculated in modified SS medium with a starting optical density of 0.2 at 600 nm, and further cultured with shaking at 36°C. Total protein was extracted with SDS-lysis buffer, and culture supernatant (CS) proteins were precipitated with 10% TCA. Protein samples were subjected to SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad) and incubated with anti-BteA, anti-BtcA, anti-BopD [32], anti-ACT, anti-FHA, or anti-PT antiserum. Antigen-antibody complexes were visualized using horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad, Hercules, CA) and ECL Western Blotting Detection Reagents (GE Healthcare).

DNA sequencing

The region between the *btcA* and *bteA* gene corresponding to positions 165122 to 168021 of *B. pertussis* Tohama (GenBank accession: BX640412) was amplified in vaccine-type and non-vaccine-type clinical strains with the appropriate primers and sequenced. Sequencing reactions were carried out with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), and the products were sequenced on an ABI PRISM 3130*xl* Genetic Analyzer (Applied Biosystems).

Transcriptional analyses

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) and treated with RNase-free DNase (Promega) to degrade contaminating DNA. Reverse transcriptase-PCR (RT-PCR) was performed with bteA RT-R and btcA RT-R primers (Table S1) using the TAKARA One Step RNA PCR Kit (AMV, TAKARA Bio Inc.). PCR was performed with the following conditions: one cycle of 50°C for 30 min, 95°C for 2 min; 25 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 1 min; and a final incubation at 72°C for 10 min. Primer sets, btcA RT-F/btcA RT-R and btcA RT-F/btcA RT-R, were used for *bteA* and *btcA* amplification, respectively (Table S1). Products were analyzed by electrophoresis on a 1.5% agarose gel. Reverse transcriptase was omitted from the negative control reaction mixtures.

For quantitative RT-PCR (qRT-PCR), 5 μg of RNA was reverse transcribed into cDNA using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA) with random hexamer primers. Relative levels of total *bteA* and *recA* transcripts were determined using TaqMan probes (bteA- and recA-probes, Table S1) and *Premix Ex Taq*™ (Perfect Real Time, TAKARA Bio Inc.) with the ABI PRISM 7500 Sequence Detection System (Applied Biosystems). The qRT-PCR conditions were 30 s at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The expression of *recA* was used as an internal control [45]. All samples were run in triplicate and *bteA* transcript (P1 + P2 transcripts) was normalized to the *recA* transcript for each sample. The *bteA* IS*481a*-promoter transcript (P2 transcript) was determined using a TaqMan probe (IS481-bteA probe). The qRT-PCR conditions were 30 s at 95°C, followed by 40 cycles of 95°C for 15 s and 55°C for 1 min. The ratio of P2 transcript to total *bteA* transcript (P2 transcript/P1 + P2 transcripts) was estimated from four independent experiments. The regions amplified by qRT-PCR are shown in Figure 6A.

Mapping transcriptional start sites

5′ rapid amplification of cDNA ends (5′-RACE) was performed using 5′-Full RACE Core Set (TAKARA Bio Inc.) according to the manufacturer's instructions. Reverse transcription was executed at 55°C using a 5′ phosphorylated RT primer (bteA-RT, Table S1). The first PCR used primers btcA-S1 (S1) and btcA-A1 (A1) primers, and bteA-S2 (S2) and bteA-A2 (A2) for the second (Table S1). PCR products were cloned into the pT7Blue T-vector (Novagen, Madison, Wis.) and transformed into *E. coli* XL1-Blue, which were plated on LB agar plates. Several clones were sequenced. The transcription start site of *btcA* was located using 5′-RACE with five primers, btcA-RT (5′ phosphorylated primer), btcA-S1 (S1), btcA-A1 (A1), btcA-S2 (S2) and btcA-A2 (A2) (Table S1).

Generation of BteA mutants

Four BteA mutants, Δ*bteA*-BP155, Δ*bteA*-BP157, ΔIS*481*-BP155 and +IS1*481*-BP157, were constructed by homologous recombination as described previously with minor modifications [30] (Figure 6A).

BteA-deficient mutants: A 2.2-kbp DNA fragment containing a 5′ portion of the *bteA* gene was amplified by PCR with the B1-btcA and B2-btcA primers (Table S1) using the *B. pertussis* Tohama genomic DNA as the template. The PCR product was cloned into the pDONR221 vector (Invitrogen) to obtain pDONR-*bteA* by means of adaptor PCR and site-specific recombination techniques with the Gateway Cloning System (Invitrogen). Inverse PCR was then carried out with R1-bteA and R2-bteA primers (Table S1) using circular pDONR-*bteA* as the template. The R1-bteA and R2-bteA primers contained a BamHI site. The resulting PCR product was digested with BamHI and self-ligated to obtain pDONR-Δ*bteA*, which contained a 178-bp deletion around the 5′ region of *bteA*. pDONR-Δ*bteA* was mixed with pABB-CRS2 [46] to obtain pABB-Δ*bteA* using the Gateway Cloning System. pABB-Δ*bteA* was then introduced into *E. coli* SM10λ*pir* and transconjugated into streptomycin (SM)-resistant *B. pertussis* BP155 (vaccine-type) and BP157 (nonvaccine-type) clinical strains. The resultant mutant strains were designated Δ*bteA*-BP155 and Δ*bteA*-BP157.

IS481-deletion mutant: pABB-*bteA* was constructed from pDONR-*bteA*. pABB-*bteA* was introduced into *E. coli* SM10λ*pir* and transconjugated into SM-resistant *B. pertussis* vaccine-type BP155. The resultant mutant strain was designated ΔIS*481*-BP155.

IS481-insertion mutant: a 3.2-kbp DNA fragment (*bteA*+IS*481*) containing the *bteA* 5′-UTR and IS*481a* was amplified with the B1-bteA and B2-bteA primers (Table S1) using *B. pertussis* BP155 genomic DNA as the template. pABB-*bteA*+IS*481* was constructed from pDONR-*bteA*+IS*481* and transconjugated into SM-resistant *B. pertussis* nonvaccine-type BP157 via *E. coli* SM10λ*pir*. The resultant mutant strain was designated +IS*481*-BP157.

Statistical analysis

The Student's *t*-test was employed. A value of $P<0.05$ was considered statistically significant.

Nucleotide sequence accession number

The IS*481a* sequence was deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession number AB473880.

Supporting Information

Figure S1 High secretion of BteA protein in *Bordetella bronchiseptica*. *B. bronchiseptica* (BB R05), *B. pertussis* BP155 (vaccine-type) and BP157 (nonvaccine-type) were cultured in modified SS medium for 24 h. Total protein extracted from the bacterial cells (Cell) and culture supernatants (CS) was separated by SDS-PAGE followed by silver staining (left panel). Immunoblots were incubated with anti-BteA, anti-BtcA or anti-BopD antiserum (right panel). For BteA detection, 0.5 μg of total protein (for Cell) and 5 μl of CS were loaded in the indicated lanes. The amount of total protein loaded was one-twentieth of that in Figure 2, and the loaded CS volume was one-fortieth of that in Figure 3.
(TIF)

Figure S2 *In vitro* transcription-translation analysis of a *bteA* 5′-UTR deletion series. (A) *bteA* 5′-UTR deletion genes were PCR-amplified using *B. pertussis* BP155 (vaccine-type) as the template. Proteins were synthesized using the WakoPURE System (Wako Pure Chemical Industries, Ltd.). The 5′-UTR deletion genes harbored the T7 promoter at their 5′ end. (B) Expression of BteA protein in an *in vitro* transcription-translation system (WakoPURE System). The synthesized product was analyzed with immunoblots using anti-BteA antiserum. NC, negative control. (C) A predicted stem-loop structure in the 5′-UTR of *bteA* mRNA (P2 transcript). The RNA secondary structure was analyzed by CentroidFold (http://www.ncrna.org/centroidfold). The schematic shows a simplified map. TIR, translation initiation region.
(TIF)

Table S1 Primers and probes in this study.
(XLS)

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Author Contributions

Conceived and designed the experiments: H-JH KK. Performed the experiments: H-JH KK. Analyzed the data: H-JH KK. Contributed reagents/materials/analysis tools: AK AA YA. Wrote the paper: KK.

References

1. Mattoo S, Cherry JD (2005) Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other *Bordetella* subspecies. Clin Microbiol Rev 18: 326–382.
2. Andrews R, Herceg A, Roberts C (1997) Pertussis notifications in Australia, 1991 to 1997. Commun Dis Intell 21: 145–148.
3. de Melker HE, Schellekens JF, Neppelenbroek SE, Mooi FR, Rümke HC, et al. (2000) Reemergence of pertussis in the highly vaccinated population of the Netherlands: observations on surveillance data. Emerg Infect Dis 6: 348–357.
4. Hellenbrand W, Beier D, Jensen E, Littmann M, Meyer C, et al. (2009) The epidemiology of pertussis in Germany: past and present. BMC Infect Dis 9: 22.
5. Tanaka M, Vitek CR, Pascual FB, Bisgard KM, Tate JE, et al. (2003) Trends in pertussis among infants in the United States, 1980-1999. JAMA 290: 2968–2975.
6. Bottero D, Gaillard ME, Fingermann M, Weltman G, Fernández J, et al. (2007) Pulsed-field gene electrophoresis, pertactin, pertussis toxin S1 subunit polymorphisms, and surfaceome analysis of vaccine and clinical *Bordetella pertussis* strains. Clin Vaccine Immunol 14: 1490–1498.
7. Fry NK, Neal S, Harrison TG, Miller E, Matthews R, et al. (2001) Genotypic variation in the *Bordetella pertussis* virulence factors pertactin and pertussis toxin in historical and recent clinical isolates in the United Kingdom. Infect Immun 69: 5520–5528.
8. Kodama A, Kamachi K, Horiuchi Y, Konda T, Arakawa Y (2004) Antigenic divergence suggested by correlation between antigenic variation and pulsed-field gel electrophoresis profiles of *Bordetella pertussis* isolates in Japan. J Clin Microbiol 42: 5453–5457.
9. Litt DJ, Neal SE, Fry NK (2009) Changes in genetic diversity of the *Bordetella pertussis* population in the United Kingdom between 1920 and 2006 reflect vaccination coverage and emergence of a single dominant clonal type. J Clin Microbiol 47: 680–688.
10. Tsang RSW, Lau AKH, Sill ML, Halperin SA, Van Caeseele P, et al. (2004) Polymorphisms of the fimbria *fim3* gene of *Bordetella pertussis* strains isolated in Canada. J Clin Microbiol 42: 5364–5367.
11. van Amersfoorth SCM, Schouls LM, van der Heide HGJ, Advani A, Hallander HO, et al. (2005) Analysis of *Bordetella pertussis* populations in European countries with different vaccination policies. J Clin Microbiol 43: 2834–2843.
12. Borisova O, Kombarova SY, Zakharova NS, van Gent M, Aleshkin VA, et al. (2007) Antigenic divergence between *Bordetella pertussis* clinical isolates from the Moscow, Russia, and vaccine strains. Clin Vaccine Immunol 14: 234–238.
13. Cassiday P, Sanden G, Heuvelman K, Mooi F, Bisgard KM, et al. (2000) Polymorphism in *Bordetella pertussis* pertactin and pertussis toxin virulence factors in the United States, 1935-1999. J Infect Dis 182: 1402–1408.
14. He Q, Mäkinen J, Berbers G, Mooi FR, Viljanen MK, et al. (2003) *Bordetella pertussis* protein pertactin induces type-specific antibodies: one possible explanation for the emergence of antigenic variants? J Infect Dis 187: 1200–1205.
15. Mooi FR, van Oirschot H, Heuvelman K, van der Heide HG, Gaastra W, et al. (1998) Polymorphism in the *Bordetella pertussis* virulence factors P.69/pertactin and pertussis toxin in The Netherlands: temporal trends and evidence for vaccine-driven evolution. Infect Immun 66: 670 675.
16. Cummings CA, Bootsma HJ, Relman DA, Miller JF (2006) Species- and strain-specific control of a complex, flexible regulon by *Bordetella* BvgAS. J Bacteriol 188: 1775–1785.
17. Shrivastava R, Miller JF (2009) Virulence factor secretion and translocation by *Bordetella* species. Curr Opin Microbiol 12: 88–93.
18. Mattoo S, Yuk MH, Huang LL, Miller JF (2004) Regulation of type III secretion in *Bordetella*. Mol Microbiol 52: 1201–1214.
19. Yuk MH, Harvill ET, Miller JF (1998) The BvgAS virulence control system regulates type III secretion in *Bordetella bronchiseptica*. Mol Microbiol 28: 945–959.
20. Coburn B, Sekirov I, Finlay BB (2007) Type III secretion system and disease. Clin Microbiol Rev 20: 535 549.
21. Ghosh P (2004) Process of protein transport by the type III secretion system. Microbiol Mol Biol Rev 68: 771–795.
22. Fennelly NK, Sisti F, Higgins SC, Ross PJ, van der Heide H, et al. (2008) *Bordetella pertussis* expresses a functional type III secretion system that subverts protective innate and adaptive immune responses. Infect Immun 76: 1257–1266.
23. Nagamatsu K, Kuwae A, Konaka T, Nagai S, Yoshida S, et al. (2009) *Bordetella* evades the host immune system by inducing IL-10 through a type III effector, BopN. J Exp Med 206: 3073–3088.
24. Medhekar B, Shrivastava R, Mattoo S, Gingery M, Miller JF (2009) *Bordetella* Bsp22 forms a filamentous type III secretion system tip complex and is immunoprotective *in vitro* and *in vivo*. Mol Microbiol 71: 492–504.
25. Caro V, Bouchez V, Guiso N (2008) Is the sequenced *Bordetella pertussis* strain Tohama I representative of the species? J Clin Microbiol 46: 2125–2128.
26. Heikkinen E, Kallonen T, Saarinen L, Sara R, King AJ, et al. (2007) Comparative genomics of *Bordetella pertussis* reveals progressive gene loss in Finnish strains. PLoS ONE 2: e904.
27. King AJ, van Gorkom T, Pennings JL, van der Heide HG, He Q, et al. (2008) Comparative genomic profiling of Dutch clinical *Bordetella pertussis* isolates using

DNA microarrays: identification of genes absent from epidemic strains. BMC Genomics 9: 311.
28. Vidakovics MLP, Paba J, Lamberti Y, Ricart CA, de Sousa MV, et al. (2007) Profiling the *Bordetella pertussis* proteome during iron starvation. J Proteome Res 6: 2518–2528.
29. Panina EM, Mattoo S, Griffith N, Kozak NA, Yuk MH, et al. (2005) A genome-wide screen identifies a *Bordetella* type III secretion effector and candidate effectors in other species. Mol Microbiol 58: 267–279.
30. Kuwae A, Matsuzawa T, Ishikawa N, Abe H, Nonaka T, et al. (2006) BopC is a novel type III effector secreted by *Bordetella bronchiseptica* and has a critical role in the type III-dependent necrotic cell death. J Biol Chem 281: 6589–6600.
31. French CT, Panina EM, Yea SH, Griffith N, Arambula DG, et al. (2009) The *Bordetella* type III secretion system effector BteA contains a conserved N-terminal motif that guides bacterial virulence factors to lipid rafts. Cell Microbiol 11: 1735 1749.
32. Nogawa H, Kuwae A, Matsuzawa T, Abe A (2004) The type III secreted protein BopD in *Bordetella bronchiseptica* is complexed with BopB for formation on the host plasma membrane. J Bacteriol 186: 3806–3813.
33. Stibitz S (1998) IS*481* and IS*1002* of *Bordetella pertussis* create a 6-base-pair duplication upon insertion at a consensus target site. J Bacteriol 180: 4963–4966.
34. DeShazer D, Wood GE, Friedman RL (1994) Molecular characterization of catalase from *Bordetella pertussis*: identification of the *katA* promoter in an upstream insertion sequence. Mol Microbiol 14: 123–130.
35. Kozak NA, Panina EM, Miller JF (2007) Type III secretion in *Bordetella* subspecies. In: Locht C, ed. *Bordetella*: molecular microbiology. Norfolk, United Kingdom: Horizon Scientific Press. pp 119 140.
36. Han H-J, Kamachi K, Okada K, Toyoizumi-Ajisaka H, Sasaki Y, et al. (2008) Antigenic variation in *Bordetella pertussis* isolates recovered from adults and children in Japan. Vaccine 26: 1530–1534.
37. Mooi FR, van Loo IHM, van Gent M, He Q, Bart MJ, et al. (2009) *Bordetella pertussis* strains with increased toxin production associated with pertussis resurgence. Emerg Infect Dis 15: 1206–1213.
38. Craig NL, Craigie R, Gellert M, Lambowitz AM (2002) Mobile DNA II. Washington DC: ASM Press. 1204 p.
39. Parkhill J, Sebaihia M, Preston A, Murphy LD, Thomson N, et al. (2003) Comparative analysis of the genome sequences of *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. Nat Genet 35: 32 40.
40. Caro V, Hot D, Guigon G, Hubans C, Arrivé M, et al. (2006) Temporal analysis of French *Bordetella pertussis* isolates by comparative whole-genome hybridization. Microbes Infect 8: 2228–2235.
41. Kaberdin VR, Bläsi U (2006) Translation initiation and the fate of bacterial mRNAs. FEMS Microbiol Rev 30: 967 979.
42. Pradel E, Guiso N, Menozzi FD, Locht C (2000) *Bordetella pertussis* TonB, a Bvg-independent virulence determinant. Infect Immun 68: 1919–1927.
43. O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250: 4007–4021.
44. Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 68: 850 858.
45. Stefanelli P, Sanguinetti M, Fazio C, Posteraro B, Fadda G, et al. (2006) Differential in vitro expression of the *brkA* gene in *Bordetella pertussis* and *Bordetella parapertussis* clinical isolates. J Clin Microbiol 44: 3397–3400.
46. Sekiya K, Ohishi M, Ogino T, Tamano K, Sasakawa C, et al. (2001) Supermolecular structure of the enteropathogenic *Escherichia coli* type III secretion system and its direct interaction with the EspA-sheath-like structure. Proc Natl Acad Sci U S A 25: 11638–11643.

百日咳

2011年から流行開始か？ ―ピークの予測は12年―

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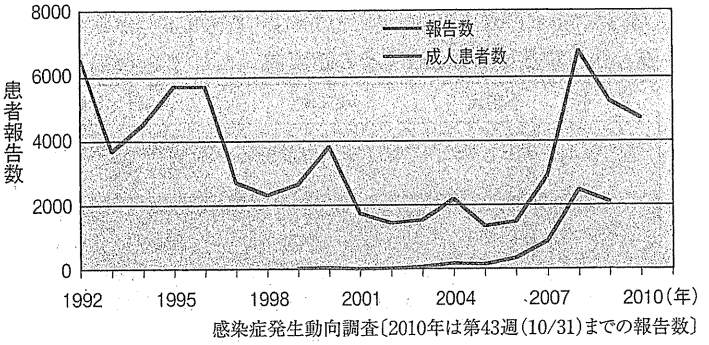
青年・成人患者が急増 先進国で進むワクチン計画見直し

百日咳は咳嗽を主訴とする細菌性の呼吸器感染症であり、ワクチン予防可能疾患(Vaccine Preventable Diseases；VPD）の一つに含まれる。患者の多くはワクチン接種前の乳児や未接種の小児であるが、近年ではワクチン効果が減弱した青年・成人層の感染が新たな問題となっている。

百日咳ワクチンの免疫持続期間は4～12年と見積もられており、多くの先進国で青年・成人患者の増加が認められている。日本でも2002年以降成人患者が急増しており、近年では報告患者数の約半数を占めるまでになった。青年・成人の保菌者は重篤化しやすい乳幼児の感染源になることが指摘されており、先進国では百日咳を再興感染症と位置付け、各国でワクチン接種プログラムの見直しを進めている。

百日咳の主な原因菌は百日咳菌（*Bordetella pertussis*）であり、患者の上気道分泌物の飛沫や直接接触により感染し、経気道的に伝播される。感染の初期段階として菌はまず上部気道に感染し、次いで気管支および小気管支の粘膜上皮細胞または繊毛間で増殖する。百日咳菌以外にヒトに感染する*Bordetella*属細菌にはパラ百日咳菌（*B. parapertussis*）、*B. holmesii*などが挙げられるが、その感染割合は低く（＜2％）、百日咳感染のほとんどが百日咳菌に由来する。百日咳菌は麻疹ウイルスと並び高い感染力を有するため、家族内感染や医療関連感染を容易に引き起こす。

図1　百日咳患者報告数の年別推移



感染症発生動向調査〔2010年は第43週(10/31)までの報告数〕

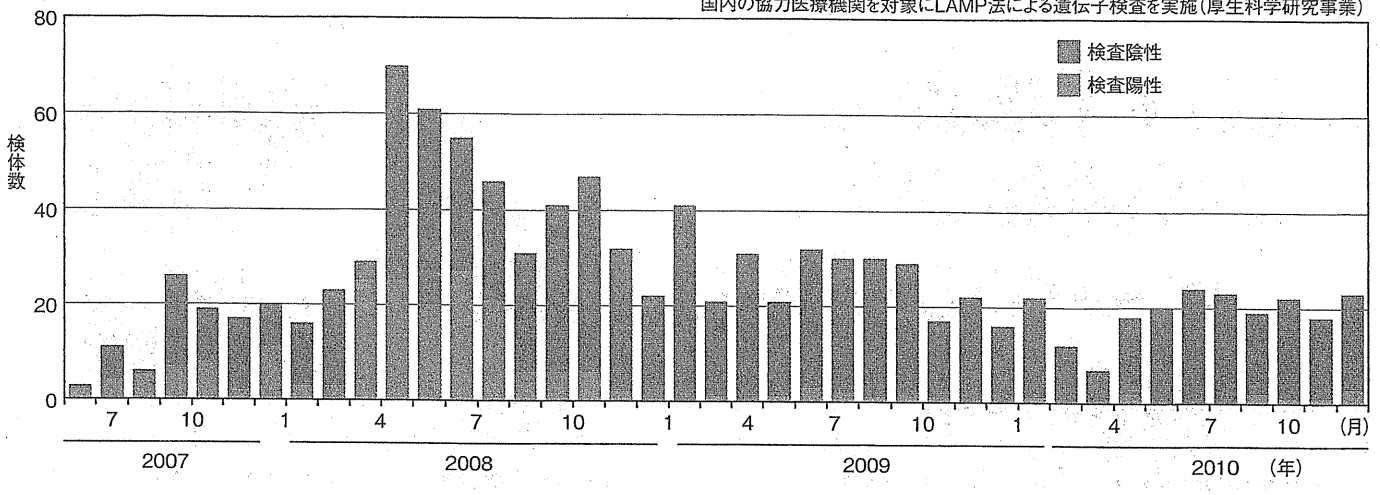
流行状況 患者報告数は他疾患含みの可能性も

百日咳は感染症発生動向調査における定点把握疾患であり、全国約3000の小児科定点から毎週患者数が報告されている。マイコプラズマ肺炎と同様に4年周期の流行を繰り返すことが知られており、近年の流行は08年に認められた（図1）。

10年第43週までの患者報告数は4682人であり、08年以降患者報告数は減少傾向を示している。一方、成人患者の割合は増加傾向を示し、08年が36.7％、09年が40.5％を占めた。この傾向は10年にも認められ、同年第24週までの成人患者は全体の51.3％を占めるまでになった。

国立感染症研究所では、厚生科学研究事業として遺伝子診断に基づく百日咳サーベイランス調査を行っている。この研究事業では、高い感度と特異性を持つloop-mediated isothermal amplification（LAMP）法を検査に採用し、07年から協力医療機関に対し病原体診断を実施している。この調査では、07年と08年に検査陽性例が多く認められたの

図2　遺伝子検査を用いた百日咳サーベイランス



に対し、09年と10年では陽性例はほとんど認められていない(**図2**)。このことは感染症発生動向調査における最近の患者報告数（09～10年）には百日咳以外の疾患が多く含まれることを示唆する。事実、最近の百日咳疑いの集団感染では百日咳菌が検出されず、マイコプラズマや夏風邪ウイルス（ライノウイルス）の遺伝子が検出されている。なお、感染症発生動向調査では主に臨床診断や血清学的診断により患者数が報告されている。

小児と成人における臨床像とその診断

百日咳に罹患した乳児は特徴的な咳嗽（吸気性笛声、痙咳発作）を示すことから、その診断は比較的容易である(**表**)。また、ワクチン未接種児は百日咳毒素による末梢白血球数の増加が認められ、臨床症状に併せて白血球ならびにリンパ球の増加が診断の一つの目安となる。一方、ワクチン既接種の年長児や青年・成人では乳児に典型的な吸気性笛声や痙咳発作が認められず、その臨床像は長期の咳といった遷延性咳嗽だけのことが多い。また、青年・成人患者の百日咳保菌量は感染初期から少ないことが知られており、菌培養検査による病原体診断はWHOでも推奨していない。保菌量の少ないワクチン既接種者、特に青年・成人では遺伝子検査に基づく病原体診断が必要である。

国外では百日咳診断に遺伝子検査が導入され始めており、米国では遺伝子検査（PCR）による診断が最も多く、次いで菌培養検査、血清学的検査（ELISA）の順となっている。日本ではまだ遺伝子検査が導入されておらず、その診断には菌凝集素価法による抗体検査が一般に用いられている。最近の報告によると、菌凝集素価法は集団単位での免疫状況を知る手段として有用であるが、感度が低いため個人レベルでの診断には適していないと結論されている。

流行対策
他者への感染防止は抗菌薬の早期投与を

前回の百日咳流行は08年に認められたことから、次回の流行は12年と予測できる。これまでの傾向から、流行は11年から始まり、そのピークは12年になるものと推察される。08年の流行は1995年以降で最も大きなものであったが、次回の流行の大きさについては予測が不可能である。これは、青年・成人患者の診断が難しいことに起因しており、08年のピークが実際の患者数を反映しているかが不明なためである。成人患者数の正確な把握には遺伝子検査に基づく病原体診断が必要であり、現在国立感染症研究所では遺伝子検査の国内での普及に努めている。近いうちに民間検査会社や病院検査室でも遺伝子検査の実施が可能となるものと期待される。

百日咳対策にはワクチン接種による免疫防御が最も効果的であり、ワクチン既接種者が感染してもその症状は軽い

表　百日咳患者の保菌量とその臨床像

患　者	保菌量	臨床像	病原体診断
乳児	極めて多い	吸気性笛声、痙咳発作	菌培養、抗体検査、遺伝子検査
年長児	多い	遷延性咳嗽(ワクチン既接種者では非典型的)	遺伝子検査(菌培養・抗体検査?)
青年・成人	少ない	遷延性咳嗽(非典型的)	遺伝子検査

ことが知られている。その一方で、ワクチン未接種の乳児が百日咳に感染すると重篤化しやすく、医療費が100万円を超す場合も珍しくない。

米国、フランスなどの先進国では成人百日咳対策として、思春期層への百日咳ワクチン追加接種が実施されている。現在、先進国のうち8カ国で追加接種が行われており、日本でも11～12歳児を対象とした追加接種が検討されている。

ワクチン以外の対策として、百日咳保菌者の早期治療が挙げられる。百日咳菌は多くの抗菌薬に感受性を示し、抗菌薬投与5日後には生菌の排出が認められなくなる。そのため、他者への感染防止には抗菌薬投与による早期治療が有効である。

早期探知、早期治療で感染拡大防止 遺伝子検査の普及に期待

07～08年の流行時には複数の大学で百日咳集団感染事例が発生し、大規模な抗菌薬の予防投薬により流行は終息した。ただし、これらの事例では早期探知ができず、感染が拡大した後で集団感染が探知された。

これらの経験を踏まえ、次回の流行時には、ワクチンによる感染予防、発症者ならびに保菌者の早期探知、抗菌薬による早期治療を徹底しなくてはならない。特に保菌者の早期探知は感染拡大防止に有効となるため、高感度な遺伝子検査法の普及が強く望まれる。 MM

医学と医療の最前線

百日咳の臨床―成人と小児―

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百日咳の臨床—成人と小児—

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要　旨

百日咳は，乳幼児の疾患と考えられてきたが，DTaPワクチン接種率向上に伴い，乳幼児の報告は激減した．近年は，相対的に10歳以上とくに成人の報告例が増加してきた．本稿では，変化してきた疫学，百日咳の家族内感染における乳幼児の症状とワクチン接種の影響，成人での症状と診断について紹介した．さらに，成人の長引く咳での百日咳の関与割合，抗原診断した成人百日咳の臨床像，感染管理，抗菌薬治療の実際および予防接種の今後の展開などを概説した．

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Key words：成人の百日咳，2週間以上の咳，百日咳の診断，予防接種

1．百日咳患者の報告数および年齢の変化

百日咳は，感染症法5類感染症・定点把握疾患に分類され，全国約3,000の小児科定点から報告されている．百日咳ワクチンを含むDTaP (Diphtheria-Tetanus-acellular Pertussis)三種混合ワクチン開始後の感染症発生動向調査での定点あたりの百日咳患者報告数を示す(図1)．1982年から4～5年ごと小さな増減をくり返しながら報告数は着実に減少してきたが，2005年から増加してきた．2007年いくつかの大学や高校での集団発生が報告され，2008年は過去10年にない多くの報告があった．

近年の特徴に患者年齢の変化がある．2000年，乳児は46.7%，1歳18.1%，2～3歳13.5%と3歳までが約80%で20歳以上は2.2%であった．次第に10～14歳以上とくに20歳以上が増加してきた．20歳以上の割合は2002年4.0%，'04年9.5%，'06年24.3%，'08年36.7%，2009年13週時点で38.2%となっている（図2)[1]．この報告は，小児科の定点医療機関に受診した患者報告である点に注意が必要である．成人は小児科でなく，内科を受診しているため，成人症例を含めた全体像を把握するためには，内科を含めた報告システムが必要となってきた．このため，国立感染症研究所感染症情報センターのホームページに，百日咳を診断した医師ならどなたでも報告できるシステムが整備されている[2]．国内の現状を把握するため，百日咳と診断された場合はご登録をお願いいたします．

2．百日咳の家族内感染

典型的な症状を呈した乳児の家族に認められた成人，同胞の症状を図3に示す．Indexは周産期に異常のない1カ月男児．軽い咳で始まり，次第にひどくなり，無呼吸・チアノーゼが認められ，百日咳（疑）で紹介入院．入院時，連続的な咳込みはあったが吸気性笛声はなかった．白血球数17,500/ul(Ly 78%) CRP＜0.30 mg/dl，百日咳菌が分離でき，典型的な乳児の百日咳と

おかだ　けんじ：国立病院機構福岡病院