TABLE 1. Incidence of Invasive Bacterial Diseases and Serotype Coverage of PCV in Hokkaido and Okinawa Prefectures Before Vaccines Introduction (2008 to 2010)

	2008		2009		2010	
	Hokkaido	Okinawa	Hokkaido	Okinawa	Hokkaido	Okinawa
Incidence (/100,000)						
Meningitis						
All-cause	8.22	11.0	9.30	14.67	7.87	15.79*
Streptococcus pneumoniae	0.97	4.89	1.96	7.33	1.97	4.86
Hib	5.80	4.89	6.36	4.89	5.90	8.50
Bacteremia						
All-cause	113.49	N/A	85.63	N/A	60.91	N/A
S. pneumoniae	65.26	97.80	57.09	80.68	58.14	89.86
Hib/Hi	36.89	13.44	17.13	23.22	2.77	12.14
Invasive pneumococcal disease	71.06	102.69	59.05	88.01	60.11	97.42
Invasive Hib diseases	42.69	18.33	17.78	40.11	8.67	20.6
PCV serotype coverage for invasive pneumococcal disease (%)						
PCV7	44	65	82	82	58	76
PCV13	78	78	82	92	94	92
Population <5 yr (Eastern Hokkaido)	206,910 (35,244)	N/A	204,247 (35,035)	81,798	203,366 (36,117)	82,353

The PCV serotype coverage for Hokkaido is based on the data from Eastern Hokkaido.

Population data are based on the annual national census.

PCV coverage for Okinawa was modified from the data in the reports referred. According to the reports, as of the end of 2010, the vaccination coverage of both Hib vaccine and PCV7 in Okinawa remained very low.

*Data not available from the original report. Calculation was based on the number of cases shown in the 2011 report per population <5 years old.

N/A indicates not available.

other developed countries, the incidence of Hib meningitis is much lower in Hokkaido, but a substantial burden of this fatal disease was observed. Again, climate/geography may play a role in the relative differences in pneumococcal and Hib incidence, but it may also be due to frequent antibiotic use.

The reported incidence of pneumococcal bacteremia in children <5 years old in prefectures other than Hokkaido and Okinawa in 2008 to 2010 was 6.5–26.1[6,7] and that of invasive Hib/Hi diseases was 11.2–24.2.[6,7] In Hokkaido, the invasive pneumococcal disease incidence was 2- to 5-fold higher and invasive Hib diseases was higher than other prefectures, with the exception of some of the more heavily populated prefectures that might have admitted patients from neighboring prefectures. Differences in the data from the mainland and island prefectures most likely arise from the considerable flow of patients across prefectural borders. Many bacteremia cases also likely go unreported in some areas.

At the end of 2011, the estimated immunization coverage of the Hib vaccine in Hokkaido Prefecture by the manufacturers was 76% and that of PCV7 was 90%, but we saw no significant reduction in incidence of meningitis. However, from January 1 to October 31, 2012, only 1 case of bacterial meningitis (by group B Streptococcus) was reported to us. According to the Hokkaido Prefecture government, utilization of the prefecture's reimbursement system indicates that as of April 2012, over 95% of children <5 years old had been vaccinated with at least 1 dose of Hib vaccine and/or PCV7 (unpublished data). Although it is too early to state any evidence-based conclusions, we expect that the Hib vaccine and PCV7 will prove highly beneficial in Japan and believe it is important to strengthen surveillance to monitor their impact.

Note: The immunization coverage of PCV7 provided by the manufacturers is calculated as follows:

Vaccination coverage = number of vaccines sold in the area / number of children <5 year old.

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RAPID DEFERVESCENCE AFTER DOXYCYCLINE TREATMENT OF MACROLIDE-RESISTANT *MYCOPLASMA PNEUMONIAE*–ASSOCIATED COMMUNITY-ACQUIRED PNEUMONIA IN CHILDREN

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Abstract: We did a retrospective review of children with *Mycoplasma pneumoniae* infection hospitalized from March 2010 to March 2013.

PLOS ONE

Genetic Analysis of *Bordetella pertussis* Isolates from the 2008–2010 Pertussis Epidemic in Japan

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Abstract

A large pertussis epidemic occurred between 2008 and 2010 in Japan. To investigate epidemic strains, we analyzed 33 *Bordetella pertussis* isolates from the epidemic period by sequencing virulence-associated genes (*fim3*, *ptxP*, *ptxA*, and *prn*) and performing multilocus variable-number tandem repeat analysis (MLVA), and compared these results with those of 101 isolates from non-epidemic, earlier and later time periods. DNA sequencing of the *fim3* allele revealed that the frequency of *fim3B* was 4.3%, 12.8%, 30.3%, and 5.1% within isolates in 2002–2004, 2005–2007, 2008–2010, and 2011–2012, respectively. The isolation rate of the *fim3B* strain therefore temporarily increased during the epidemic period 2008–2010. In contrast, the frequencies of the virulence-associated allelic variants, *ptxP3*, *ptxA1*, and *prn2*, increased with time during overall study period, indicating that these variants were not directly involved in the occurrence of the 2008–2010 epidemic. MLVA genotyping in combination with analysis of allele types showed that the prevalence of an MT27d strain temporarily increased in the epidemic period, and that this strain carried virulence-associated allelic variants (*fim3B*, *ptxP3*, *ptxA1*, and *prn2*) also identified in recent epidemic strains of Australia, Europe, and the US. Phenotypic analyses revealed that the serotype Fim3 strain was predominant (≥87%) during all the periods studied, and that the frequency of adhesion pertactin (Prn) non-expressing *B. pertussis* decreased by half in the epidemic period. All MT27d strains expressed Prn and Fim3 proteins, suggesting that *B. pertussis* MT27d strains expressing Prn and Fim3B have the potential to cause large epidemics worldwide.

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Introduction

Bordetella pertussis, a highly communicable Gram-negative coccobacillus, is the cause of pertussis (whooping cough), a major acute respiratory infection resulting in severe childhood illness and infant death [1]. Although universal immunization programs have contributed to significant reductions in the morbidity and mortality rates associated with pertussis, the incidence of pertussis has increased in several countries despite high vaccination coverage [2-6]. In Japan, acellular pertussis vaccines (ACVs) were introduced in 1981 and are used to control pertussis with a schedule of 3 primary doses and single booster dose at ages 3, 4, 6, and 18−23 months, respectively. This vaccination schedule has been followed since 1994. The incidence of pertussis cases in adolescents and adults, however, has significantly increased since the early 2000s [7]. The waning of vaccine-acquired immunity and the decrease in opportunities of natural immune boosting owing to reduced levels of *B. pertussis* circulation have been proposed as explanations for the recent resurgences of pertussis [2,8,9]. Another possible underlying factor is the adaptation of the *B. pertussis* population to vaccine-induced immunity [2,10,11].

Antigenic and genetic shifts in *B. pertussis* circulating strains have been identified within virulence-associated genes encoding serotype 3 fimbriae (*fim3*), pertussis toxin S1 subunit (*ptxA*), pertactin (*prn*), and the pertussis toxin promoter (*ptxP*). Allele frequencies of the virulence-associated allelic variants, *fim3B*, *ptxA1*, *prn2*, and *ptxP3*, have increased within the *B. pertussis* population in several countries [10,12–17]. *B. pertussis* strains carrying *ptxP3* are more capable of producing pertussis toxin (PT) than are *ptxP1* strains, and the emergence of *ptxP3* strains was associated with pertussis resurgence in the Netherlands [18]. Similarly, a significant correlation was observed between an increase in *fim3B* strains and pertussis notifications in the US [13]. Strains with *fim3B* have a single amino-acid substitution (A87E) within the surface epitope of Fim3, which interacts with human serum [19,20]. Furthermore, multilocus variable-number tandem repeat analysis (MLVA) has revealed that the *B. pertussis* population has changed over the past 50 years worldwide. In Australia, the frequency of *B. pertussis* MLVA type 27 (MT27) and MT70 strains increased after the introduction of an ACV, and subsequently, the MT27 strain became predominant in 2008−2010 [21,22]. An increase in the frequency of *B. pertussis* MT27 strain was also observed in Europe and the US [13,16,23].

Pertussis epidemics still occur worldwide, and epidemic strains have been characterized by Fim serotyping and/or genotyping within some regions [24–26]. In a Dutch epidemic, significant changes in Fim serotypes and MTs were observed during a period when the pertussis vaccine dose was lowered [24]. Besides phenotypic variants of Fim, *B. pertussis* variants that do not express adhesion pertactin (Prn) have been recently identified in Japan as

well as in other countries [27–30]. Since Prn is a component of ACVs, it is reasonable to hypothesize that Prn-negative variants have increased fitness in humans immunized with ACV. To date, the relationship between the prevalence of Prn-negative variants and pertussis epidemics has not been evaluated.

In Japan, a large pertussis epidemic occurred in 2008−2010 despite high vaccination coverage with ACVs. To elucidate the causes of the epidemic, we determined temporal trends in the frequencies of virulence-associated genes (*fim3*, *ptxP*, *ptxA*, and *prn*) and genotypes in the *B. pertussis* population from 2002 to 2012. In addition, phenotypes of epidemic isolates were characterized by their expression of Fim and Prn proteins.

Materials and Methods

Pertussis surveillance data

National surveillance data were obtained from the Ministry of Health, Labor and Welfare of Japan Infectious Disease Surveillance data. Each week, the number of pertussis cases was reported from approximately 3,000 sentinel clinics and hospitals within Japan. Diagnosis was based on bacterial culture, clinical symptoms, and/or the results of a serologic test. The reporting criteria did not change during the study period, 2002−2012.

Bacterial strains

We examined 134 clinical *B. pertussis* isolates collected in Japan from 2002 to 2012 (Table S1). Thirty-three of those isolates were collected during the 2008–2010 pertussis epidemic, while 101 isolates were collected from non-epidemic periods: 23 in 2002–2004, 39 in 2005–2007, and 39 in 2011–2012 (Fig. 1). All isolates were epidemiologically unrelated cases of pertussis. The isolates were cultured on Bordet-Gengou agar (Difco) or cyclodextrin solid medium (CSM) agar [31], and incubated at 36°C for 2−3 days. DNA was extracted from *B. pertussis* isolates by boiling, and stored at −20°C.

Sequence analysis of *fim3*, *ptxP*, *ptxA*, and *prn*

Four virulence-associated genes, *fim3*, *ptxP*, *ptxA* and *prn*, were analyzed using PCR-based sequencing [14,18,32,33]. Sequence reactions were carried out with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems), and resultant products were sequenced using an Applied Biosystems 3130xl Genetic Analyzer or 3730 DNA Analyzer. Subsequent sequencing of the variable region 2 (R2) of *prn* was performed where necessary, to distinguish between *prn1* and *prn7* alleles. Primer sets used in this study are listed in Table S2.

MLVA

MLVA typing was performed as previously described [22,28]. MTs were assigned using the MLVA typing tool found at http://www.mlva.net. Novel MLVA types were assigned by the webmasters, Drs. H. van der Heide and F. Mooi, from the Centre for Infectious Disease Control Netherlands, within National Institute for Public Health and the Environment, in the Netherlands.

Immunoblotting and serotyping analysis

Prn expression in *B. pertussis* isolates was analyzed by immunoblotting [28]. Briefly, protein samples (1 μg) were first subjected to 10% SDS-PAGE, then transferred onto nitrocellulose membranes (Bio-Rad), and finally incubated with anti-Prn antiserum. Antigen-antibody complexes were visualized using a horseradish peroxidase-conjugated secondary antibody (Bio-Rad) and the Western Lightning ECL Pro (PerkinElmer, Inc.). Resultant blots were imaged using a LAS-3000 (Fujifilm, Tokyo, Japan).

Serotyping of *B. pertussis* isolates was performed with indirect whole-cell ELISA using anti-Fim2 and anti-Fim3 monoclonal antibodies as previously described [34,35], with some minor modifications. Briefly, bacterial cells cultured on Bordet-Gengou agar were resuspended in phosphate-buffered saline (PBS) to an optical density of 0.01 at 620 nm, and then inactivated at 56°C for 1 h. The wells of 96-well ELISA plate (Nunc Immuno Plate Maxisorp) were coated with 100 μl of this suspension to each well and allowing it to evaporate overnight at 36°C. Anti-Fim2 (NIBSC 04/154) and anti-Fim3 (NIBSC 04/156) antibodies were used at a 1:1,000 dilution in PBS. Antibody binding to bacterial cells was detected following the addition of a 1:4,000 dilution of alkaline phosphatase-labeled goat anti-mouse IgG (Southern Biotechnology Associates, Inc.) and with the use of *p*-nitrophenylphosphate as a substrate. The optical density was measured at 405 nm with 650 nm as a reference using a Multiskan FC microplate reader (Thermo Fisher Scientific Inc.). *B. pertussis* strain 18323 that expresses both Fim2 and Fim3 was used as a positive control.

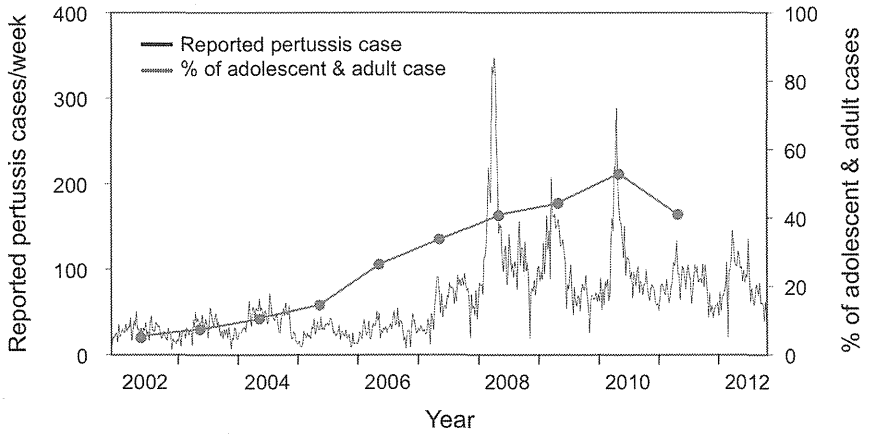

Figure 1. Number of reported pertussis cases per week in Japan from 2002 to 2012. Pertussis cases are shown by the black line, with each value representing a week of the year. The percentage of adolescent and adult cases (≥15 years old) per year is shown in red circles. The data were obtained from the Ministry of Health, Labor and Welfare of Japan Infectious Disease Surveillance data. Data regarding the number of adolescent and adult cases in 2012 were not available.
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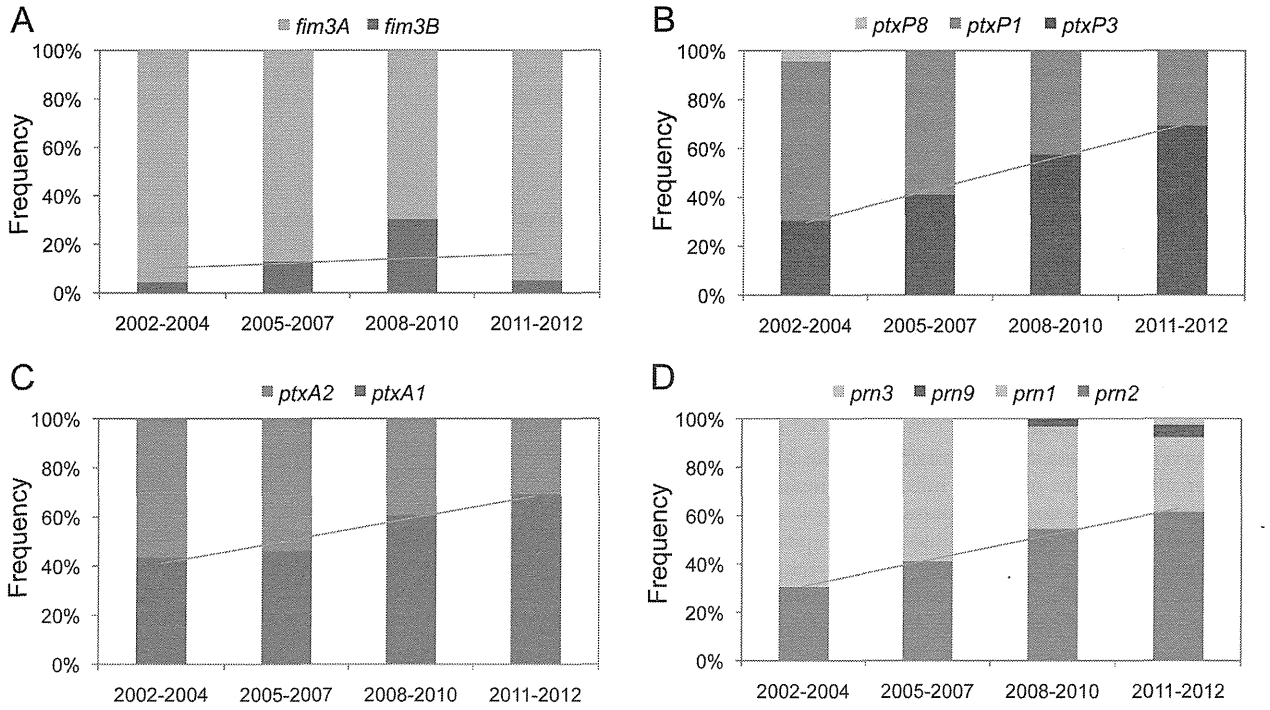

Figure 2. Temporal trends in the frequencies of *fim3*, *ptxP*, *ptxA*, and *prn* alleles within *Bordetella pertussis* isolates in Japan from 2002 to 2012. Four allelic genes, *fim3* (A), *ptxP* (B), *ptxA* (C), and *prn* (D), of 134 *B. pertussis* isolates were sequenced. Isolate allele frequencies are shown by time period: 2002−2004 (non-epidemic, n = 23), 2005−2007 (pre-epidemic, n = 39), 2008−2010 (epidemic, n = 33), and 2011−2012 (post-epidemic, n = 39). The regression line shows the relationship between the frequency of virulence-associated allelic variant (*fim3B*, *ptxP3*, *ptxA1*, or *prn2*) and the 4 time periods.
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Statistical analysis

Squared Pearson's correlation coefficient (R^2) was used to identify a linear dependence between allele frequency (*fim3B*, *ptxP3*, *ptxA1*, or *prn2*) and isolation periods. Fisher's exact test was performed to analyze the distribution of *B. pertussis* population. The Simpson's diversity index (DI) and 95% confidence interval (CI) of MTs was calculated as described by Hunter and Gaston [36] and Grundmann et al. [37], respectively, using the online tool available at http://www.comparingpartitions.info/.

Results

Characteristics of the 2008−2010 pertussis epidemic in Japan

There were 17,349 reported pertussis cases between January 2008 and December 2010 in Japan. Within this pertussis epidemic period, 3 sharp peaks representing increases in case frequency were observed: 1 in late May 2008 (347 cases at week 22), 1 in mid-May 2009 (207 cases at week 20), and 1 in mid-June 2010 (289 cases at week 24) (Fig. 1). The number of reported cases per year in 2008−2010 was ≥2.7 times higher than the previous 5-year average. Although the number of pertussis patients over 15 years of age steadily increased in the 2000s, alongside increases of adolescent and adult incidence rates (40.7%, 44.2%, and 52.9% of all reported cases in 2008, 2009, and 2010, respectively), in 2011, the number of those patients decreased and the incidence rate in adolescents and adults also decreased to 41%.

Temporal changes in the frequencies of *fim3*, *ptxP*, *ptxA*, and *prn* alleles

Among the 134 *B. pertussis* isolates tested, 2 *fim3* (*fim3A* and *fim3B*), 3 *ptxP* (*ptxP1*, *ptxP3* and *ptxP8*), 2 *ptxA* (*ptxA1* and *ptxA2*), and 4 *prn* (*prn1*, *prn2*, *prn3*, and *prn9*) alleles were identified. Figure 2 shows the temporal trends of the allele frequencies. The frequency of the allele *fim3B* temporarily increased during the epidemic period (2008–2010): it was 4.3% in 2002–2004, 12.8% in 2005–2007, 30.3% in 2008–2010, and 5.1% in 2011–2012 (Fig. 2A). In contrast, the frequencies of *ptxP3*, *ptxA1*, and *prn2* increased with time from 2002 to 2012 (Fig. 2B-D). High correlations ($R^2 \geq 0.95$) were observed between these latter allele frequencies and the isolation periods.

Temporal changes in the frequencies of Prn and Fim variants

The Prn and Fim expression phenotypes of the 134 *B. pertussis* study isolates were determined with immunoblotting and indirect whole-cell ELISA, respectively. Figure 3A shows the temporal trend of the frequencies of the 2 identified Prn variants, Prn-expressing and Prn-negative strains, with the frequency of Prn-negative strains at 43.5%, 41.0%, 21.2%, and 25.6% during 2002–2004, 2005–2007, 2008–2010, and 2011–2012, respectively. A decreased frequency of Prn-negative strains was observed during the epidemic period (2008–2010); however, this decrease was not statistically significant ($P>0.05$). All the Prn-negative strains carried the *prn1* allele (Table S1). Prn-negative strains carrying *prn1* were previously found in Finland [30], and those carrying *prn2* were found in France and the US [27,29].

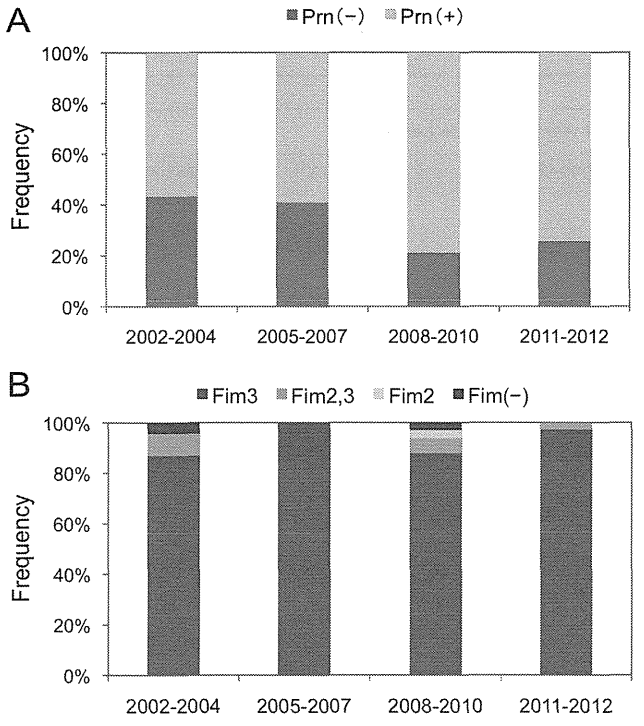

Figure 3. Temporal trends in the frequencies of Prn and Fim3 variants of *Bordetella pertussis* isolates in Japan from 2002 to 2012. Prn (A) and Fim (B) expression was analyzed within 134 *B. pertussis* isolates. Two Prn variants, Prn(+) and Prn(−), and 4 Fim variants, Fim2, Fim3, Fim2,3, and Fim(−) strains, were identified. Variant frequencies are shown by time period: 2002−2004 (non-epidemic, n = 23), 2005−2007 (pre-epidemic, n = 39), 2008−2010 (epidemic, n = 33), and 2011−2012 (post-epidemic, n = 39).
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On the other hand, 4 Fim variants, Fim2, Fim3, Fim2,3, and Fim(−), were identified among the *B. pertussis* isolates. The Fim2,3 strain was detected by both Fim2 and Fim3 antigens, while the Fim(−) strain was not detected by either. As shown in Figure 3B, the Fim3 strain was predominant during all the time periods studied: 87.0% in 2002−2004, 100% in 2005−2007, 87.9% in 2008−2010, and 97.4% in 2011−2012. Two Fim(−) strains were isolated in 2002−2004 and 2008−2010. Interestingly, one Fim(−) strain was previously identified in Ontario, Canada [12].

Temporal changes in MTs and genetic diversity

Among the 134 *B. pertussis* study isolates, 24 different MTs were identified, of which 2 were novel (MT251 and MT253). Figure 4 shows minimum spanning trees that revealed the genetic diversity of the *B. pertussis* population during the time periods of 2002−2004, 2005−2007, 2008−2010, and 2011−2012. Eighteen *B. pertussis* isolates carrying *fim3B* were identified during the 4 time periods, and these *fim3B* strains were divided into 4 MTs: MT26 (n = 1), MT27 (n = 14), MT32 (n = 1), and MT69 (n = 2). Although MT27 and MT186 were the predominant types during all the time periods, the *fim3B* strain did not belong to MT186. MT27 strains carrying *fim3B* were most frequent in MT27 during the epidemic period (2008−2010) at 56% (10/18), and their frequency decreased to 7% (2/27) in 2011−2012. The temporal increase of MT27 strains carrying *fim3B* was statistically significant ($P < 0.05$) when compared with non-MT27 strains carrying *fim3B*.

MT27 strains could be further classified into 5 subtypes (MT27a to MT27e) based on their allele types (Table 1). MT27a strains

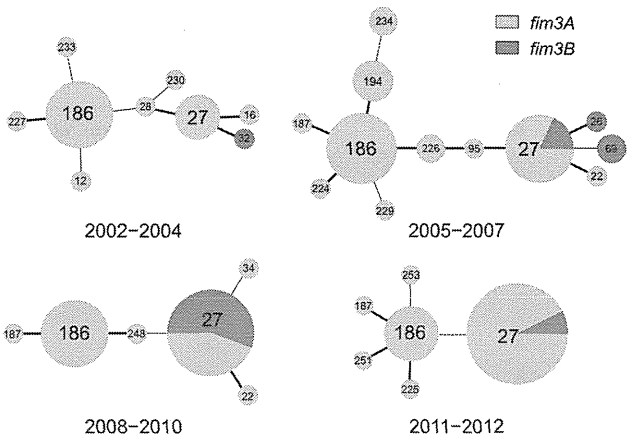

Figure 4. Minimum spanning trees revealing the genetic diversity of the *Bordetella pertussis* population in Japan from 2002 to 2012. MTs were determined for 134 *B. pertussis* isolates. The resultant phylogenetic trees based on MTs are shown by time period: 2002−2004 (non-epidemic, n = 23), 2005−2007 (pre-epidemic, n = 39), 2008−2010 (epidemic, n = 33), and 2011−2012 (post-epidemic, n = 39). Each circle within a tree represents a different MT, with the MT number noted. Thick lines separate single-locus variants, while thin lines separate double-locus variants, and dotted lines signify a more distant relationship. Pink and red colors indicate, respectively, the *fim3A* and *fim3B* allele frequencies within MTs.
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carried *fim3A*, *ptxP3*, *ptxA1*, and *prn2*, and were the predominant subtype. Notably, they were collected in both non-epidemic and epidemic periods. In contrast, MT27d strains carrying *fim3B*, *ptxP3*, *ptxA1*, and *prn2* were predominantly isolated during the epidemic period, with 10 of 12 isolates (83%) being of this subtype in 2008−2010. An MT27c strain carrying *fim3A*, *ptxP3*, *ptxA1*, and *prn9* was also isolated during the epidemic period. The other MT27 subtypes, MT27b and MT27e, were found only in 2011−2012.

The genetic diversity of MTs and the 5 MT27 subtypes was subsequently determined. As shown in Figure 5, Simpson's DI was 0.74 (95% CI, 0.58−0.90), 0.85 (0.78−0.92), 0.77 (0.70−0.84), and 0.59 (0.43−0.75) in 2002−2004, 2005−2007, 2008−2010, and 2011−2012, respectively. Therefore, the genetic diversity within *B. pertussis* population decreased after the 2008−2010 pertussis epidemic.

Relationship between MTs and phenotypes in 2008−2010

During the 2008−2010 pertussis epidemic, MT186 (33%), MT27a (21%), and MT27d (30%) were the predominant MTs, and most of them were serotype Fim3 strains (89%) (Table 2). Fim3 strains were also identified within other minor MTs (MT22, MT27c, MT187, and MT248), while a Fim2 strain belonged to MT34, the 2 Fim2,3 strains were typed as MT27d and MT186, and the Fim(−) strain belonged to MT27a. Of the 10 MT27d strains, 9 expressed Fim3 and 1 expressed both Fim2 and Fim3. On the other hand, Prn-negative strains were all observed within MT186. Seven (64%) of the 11 MT186 strains did not express Prn. All other MTs expressed Prn.

Discussion

In the present study, we demonstrated that the prevalence of *B. pertussis* strains carrying *fim3B*, which were classified as MT27d, temporarily increased during the 2008–2010 pertussis epidemic in

Table 1. Comparison of MTs and allele types between *Bordetella pertussis* isolates collected in non-epidemic and epidemic periods.

	Allele types				No. of isolates detected within the time period			
MT	*fim3*	*ptxP*	*ptxA*	*prn*	2002–2004	2005–2007	2008–2010*	2010–2012
12	A	8	2	1	1			
16	A	3	1	2	1			
22	A	3	1	2		1	1	
26	B	3	1	2		1		
27a	A	3	1	2	5	9	7	24
27b	A	3	1	3				1
27c	A	3	1	9			1	
27d	B	3	1	2		2	10	
27e	B	3	1	9				2
28	A	1	1	1	1			
32	B	3	1	2	1			
34	A	1	1	1			1	
69	B	3	1	2		2		
95	A	3	1	2		1		
186	A	1	2	1	11	12	11	8
187	A	1	2	1		1	1	1
194	A	1	2	1		4		
224	A	1	2	1		1		
225	A	1	2	1				1
226	A	1	2	1		2		
227	A	1	2	1	1			
229	A	1	2	1		1		
230	A	1	1	1	1			
233	A	1	1	1	1			
234	A	1	1	1		2		
248	A	1	2	1			1	
251	A	1	2	1				1
253	A	1	2	1				1

*Pertussis epidemic period.

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Japan. The MT27d strains of the epidemic period were collected in several areas (Table S1). All MT27d strains carried *fim3B*, *ptxP3*, *ptxA1*, and *prn2*, and expressed Prn and Fim3 proteins. Although Prn-negative strains have recently increased in their prevalence within Japan and in other countries [27–30], here, a lowered frequency of Prn-negative strains was observed during the epidemic period specifically, indicating that Prn-negative strain types were not involved in the epidemic. Similarly, we found that the prevalence of the virulence-associated allelic variants, *ptxP3*, *ptxA1*, and *prn2*, has increased with time from the early 2000s, indicating that the variants were also not directly associated with the epidemic. Taken together, our findings demonstrate that *B. pertussis* strains carrying *fim3B* (i.e., MT27d) were associated with the 2008–2010 pertussis epidemic.

We evaluated the regional effect on *B. pertussis* population because of the low number of samples of isolates. When the number of isolates was compared, the difference in regional population between epidemic (2008–2010) and post-epidemic (2011–2012) periods was statistically significant ($P < 0.01$), possibly because of the high number (18/39) of isolates collected in the Kinki district (including Osaka) during the post-epidemic period. A sampling bias cannot be excluded from the analysis of the trend in 2011–2012. However, no significant difference was observed between pre-epidemic (2005–2007) and epidemic periods ($P > 0.05$). The regional effect was therefore small or negligible for detection of the emergence of strain MT27d in the 2008–2010 epidemic.

In the past decade, the prevalence of *B. pertussis* strains carrying *fim3B* has increased worldwide [12–14,25]. In Ontario, Canada, 1 predominant *B. pertussis* clone was identified in the 2000s [12]. This strain carried the same virulence-associated allelic variants (*fim3B*, *ptxP3*, *ptxA1* and *prn2*) as our epidemic strains within MT27d and was identical to the strains involved in recent pertussis resurgences within Europe and Australia. Similarly, *fim3B* strains carrying *ptxP3*, *ptxA1*, and *prn2* were predominant in the US during the 2000s, and most were genotyped to MT27 [13]. Interestingly, the pertussis resurgence in the US was correlated with the emergence and predominance of the *fim3B* allele, but not with the *ptxP3* allele. On the other hand, in the Netherlands, the prevalence of *fim3B* strains temporarily increased in the early

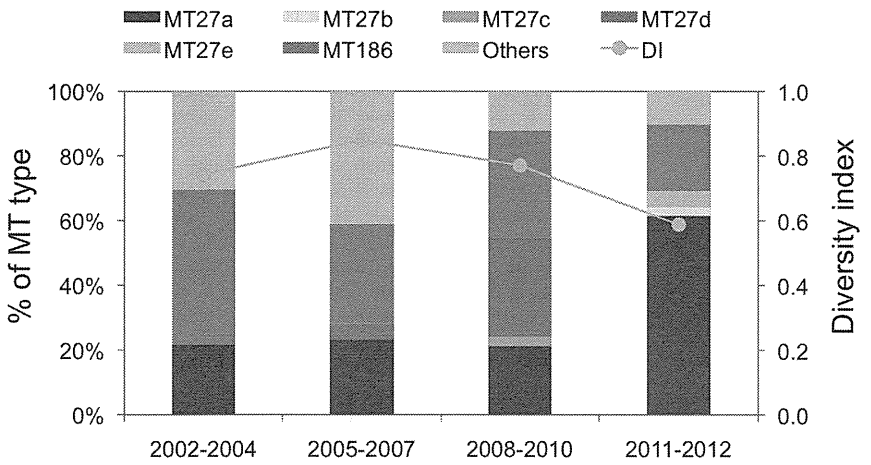

Figure 5. Frequency of MLVA types and genetic diversity of the *Bordetella pertussis* population in Japan from 2002 to 2012. The diversity index (DI) and 95% confidence interval (CI) were calculated from the MT frequencies within 4 time periods: 2002–2004 (non-epidemic), 2005–2007 (pre-epidemic), 2008–2010 (epidemic), and 2011–2012 (post-epidemic). For convenience, minor MTs (MT22, MT34, MT187, and MT248) are shown as "Others".
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2000s, although the strains disappeared in 2010 [14]. Likewise, MT27d strains (carrying *fim3B*) were not identified after the 2008–2010 epidemic period, and the reasons behind the disappearance of this strain type are unclear. Our findings along with those of previous studies, therefore, suggest that the MT27d strain is a recent epidemic strain that is found worldwide, and that this strain is not only associated with pertussis resurgence but can also be correlated with pertussis epidemics.

Fimbriae of *B. pertussis* are composed of Fim2 and/or Fim3 and FimD. The minor fimbrial subunit FimD forms the adhesive tip [38]. Fim2 and Fim3 are encoded by the single-copy genes *fim2* and *fim3*, respectively [39,40], and are serologically distinct [41]. Fim resulting from the *fim3B* strain is Fim3B, which differs from Fim3A by a single amino-acid substitution (A87E) at the surface epitope. To date, the biological differences between Fim3A and Fim3B are unknown. In an effort to address this issue, we recently observed *B. pertussis* clinical strains and found that most strains carrying *fim3B* had strong autoagglutination capability, unlike those carrying *fim3A*, following the suspension of CSM agar cultures into saline (Otsuka and Kamachi, unpublished data).

Table 2. Relationship between MTs and phenotypes in *Bordetella pertussis* isolates collected during the 2008–2010 pertussis epidemic period.

		Fim expression				Prn expression	
MT	No. of isolates	Fim2	Fim3	Fim2,3	Fim(−)	Prn(+)	Prn(−)
22	1		1			1	
27a	7		6		1	7	
27c	1		1			1	
27d	10		9	1		10	
34	1	1				1	
186	11		10	1		4	7
187	1		1			1	
248	1		1			1	

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Surprisingly, autoagglutination was not observed when the strains were cultured on Bordet-Gengou agar. Bacterial autoaggregation is a phenomenon associated with pathogen virulence in many Gram-negative bacteria [42–46], suggesting Fim3B strains are more virulent than Fim3A strains because of their ability to autoagglutinate. Further study is necessary to fully elucidate the relationship between autoagglutination and Fim3B. Attempts to identify the molecular mechanism that regulates autoagglutination within *fim3B* strains are currently underway.

In many countries, a shift from serotype Fim2 to Fim3 in *B. pertussis* circulating strains was observed after mass vaccination, and antigenic differences (in Fim, PT, and Prn) have been since noted between *B. pertussis* vaccine strains and circulating strains. In Japan, the *B. pertussis* strain Tohama carrying *ptxA2*, *prn1*, and *fim2* has been used as a vaccine strain to produce ACVs from 1981. Among the 4 currently used Japanese ACVs, 2 contain Fim2 and all do not contain Fim3 [47]. A recent study demonstrated that Fim2 and Fim3 are immunogenic antigens, and that individuals recently infected with pertussis had greater anti-Fim3 IgG concentrations than anti-Fim2 IgG concentrations, consistent with the current predominance of Fim3 strains [41]. Based on this finding, there is a clear need for the improvement of currently used ACVs. Specifically, the inclusion of Fim3 (Fim3A and/or Fim3B) in ACVs may be an effective way to reduce the number of current circulating *B. pertussis* strains, including Fim3B strains. Although Fim2 has been shown to be a protective antigen, the protective immunogenicity of Fim3 is still unknown [48]. Further study of this topic will be required to evaluate the effectiveness of Fim3 as a protective antigen.

In the present study, the genetic diversity of the *B. pertussis* population decreased after the 2008–2010 pertussis epidemic. This decrease reflects the expansion of the MT27a strain type and the disappearance of MT27d strains. The MT27a strains carried the same *ptxP3*, *ptxA1*, and *prn2* alleles as the MT27d strains, but additionally carried *fim3A*. Significant changes in the *B. pertussis* population were previously observed in a pertussis epidemic in the Netherlands, and this study suggested that strain surveillance may serve as an early detector of pertussis epidemics [24]. Here, we observed significant changes within the *B. pertussis* population during the 2008–2010 epidemic, lending further support for an early warning system of future pertussis epidemics.

In conclusion, the prevalence of *B. pertussis* MT27d strains temporarily increased during the 2008−2010 pertussis epidemic in Japan. The MT27d strains carried the same virulence-associated allelic variants (*fim3B*, *ptxP3*, *ptxA1*, and *prn2*) as recent epidemic strains observed in Australia, Europe, and the US. *B. pertussis* MT27d strains, therefore, likely have the potential to cause large epidemics in other countries, and, hence, further study and strain surveillance of the MT27d strain type is warranted.

Supporting Information

Table S1 Characteristics of *Bordetella pertussis* study isolates.
(XLSX)

Table S2 PCR primers in this study.
(XLSX)

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Author Contributions

Conceived and designed the experiments: YM NO KK . Performed the experiments: YM NO HT-A. Analyzed the data: YM NO KK. Contributed reagents/materials/analysis tools: NO KS KK. Wrote the paper: YM KK.

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PLOS ONE

A New Method for the Detection of Neutralizing Antibodies against Mumps Virus

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Abstract

Neutralization test is the most reliable method of evaluating immunity against viral diseases but there is no standard procedure for mumps virus, with tests differing in the infectivity of the challenge virus, 50% plaque reduction or complete inhibition of cytopathic effects (CPE), and usage of complement. A reliable, easy, and simple neutralization test for mumps virus was developed in this study. A recombinant mumps virus expressing GFP was generated as a challenge virus. Complement was added to the neutralizing mixture at 1:200 when stocked serum samples were used. Neutralizing antibody titers were expressed as the reciprocal of the highest dilution that did not exceed two-fold of FU values (GFP expression) of the cell control wells. A total of 1,452 serum samples were assayed by inhibition of GFP expression in comparison with those examined by conventional 100% inhibition of CPE. 1,367 (94.1%) showed similar neutralizing antibody titers when examined by both methods. The GFP expression inhibition assay, using a recombinant mumps virus expressing GFP, is a simple and time- saving method.

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Introduction

Mumps virus is a single-stranded negative sense RNA virus, belonging to the genus *Rubulavirus* of the family *Paramyxoviridae*. The mumps virus genome encodes seven major proteins in the following gene order: nucleocapsid (N), phospho (P), matrix (M), fusion (F), small hydrophobic (SH), hemagglutinin-neuraminidase (HN), and large (L) protein genes [1]. V and I proteins are also produced from the P gene. There are two envelope glycoproteins, F and HN. The HN protein is involved in the virus attachment to sialic acid receptors on the surface of host cells. This leads to a conformational change of HN which induces further conformational change of the F protein in the cascade reaction of cell fusion [1,2]. Thus, mumps virus infection is initiated by the F and HN proteins, and neutralizing epitopes are located on these proteins [3,4].

An acute infection of mumps virus is characterized by self-limiting demonstrable swelling of the parotid glands with tenderness and several complications have been reported following parotitis, including aseptic meningitis, deafness, orchitis, and pancreatitis [1,5]. Mumps virus circulates throughout the world, and genotype classification of the wild type is useful for identifying the pathway of transmission [6]. Recently, circulating mumps virus strains have been divided into 12 genotypes from A to N (excluding E and M) based upon the sequence diversity of the SH gene [7,8]. Currently circulating strains in Japan were divided into four genotypes, B, G, J, and L [9].

The isolation of mumps virus is essential for the diagnosis of patients and for monitoring the antigenicity of wild circulating strains. The efficiency of virus isolation depends mainly upon the infectious viral load in clinical samples and the sensitivity of the cells used for isolation. Vero cells have been used, but isolation is not always successful because of the low viral load, timing of sample taking, and transportation. Several serological tests have been employed for the diagnosis of mumps virus infections and, among them, the enzyme-linked immuno-assay (EIA) was used to detect IgM antibodies for diagnosis and IgG EIA to investigate immune status [10,11]. EIA antibodies did not reflect protective immunity and a neutralization test is the most sensitive way to predict protective immunity [12,13]. Neutralization tests take a long time to obtain results and involve several complicated procedures [14,15]. The sensitivity of neutralization test was enhanced when complement was added [15]. Recently, the addition of complement was found to lead to deposition on the surface of viral particles bound with antibodies and destroyed the structure of mumps virus during the neutralization reaction [16]. Thus, the presence of complement seems to be essential for neutralization testing against mumps virus. In this study, a recombinant mumps virus expressing green fluorescent protein (GFP) was generated and the requirement for complement was examined using fresh and stocked sera.

Materials and Methods

Mumps Virus Strain

The Hoshino vaccine seed strain KO3 was developed by attenuation through 22 passages in chick embryonic cells from a wild-type mumps virus isolated in 1972 [17]. Full-length cDNA was constructed from KO3 Hoshino. The GFP sequence was inserted between the P/V and M genes (Fig. 1). GFP Hoshino was recovered from 293 T cells transfected with N, P, and L expression plasmids, and full-length cDNA under the control of T7 RNA polymerase [18]. Monolayer of Vero cells was infected with GFP Hoshino at m.o.i = 0.01 and culture fluid was stocked for challenge virus.

Virus Infectivity

Vero cells were propagated in minimum essential medium (MEM) supplemented with 5% fetal bovine serum (FBS). Infectivity was determined based on the $TCID_{50}$ in Vero cells. The virus culture fluid was serially diluted by 10-fold and a confluent monolayer of Vero cells was infected with 100 µl of each dilution in 96-well plates. The plates were incubated for 2 h at 37°C in 5% CO_2 and MEM supplemented with 2% FBS was added. Infectivity was determined after incubation for 7 days.

Serum Samples

Eight serum samples obtained from healthy adults aged 23 to 58 years during a routine health check were used for the experiments after obtaining verbal informed consent. The remaining portion of the sera was used for preliminary experiments or as in-house control serum. Stocked serum samples (n = 185) were obtained to assess immunity against measles, mumps, rubella, and chickenpox among new students of the nursing school of Ashikaga Red Cross Hospital, Tochigi prefecture. The serological study was approved by the ethics committee of the hospital and verbal informed consent was obtained. Fresh serum samples (n = 1,452) obtained to evaluate immunity against measles, mumps, rubella, and chickenpox among new students in primary, junior high, and high schools, were used for routine yearly immunological assessments of infection control and to advise regarding vaccination for antibody negative pupils. The serological study was approved by the Health Care Center of Keio University. The purpose of the study was explained and written informed consent was obtained from their guardians. Serum samples were anonymously transferred to our laboratory, labeled with simplified numbers.

Virus Neutralization Test

The fresh serum samples were divided into several aliquots and stocked at −20°C. The samples were kept at 56°C for 30 min to inactivate the complement, serially diluted by 2-fold starting from 1:4, and mixed with the same volume of GFP Hoshino containing 100 $TCID_{50}$ of infectious virus at 37°C for 90 min for neutralization. The mixture was placed in 96-well plates in duplicate for each dilution and 25,000 Vero cells were seeded in 0.1 ml. The plates were incubated for 7 days. In order to calculate the titers automatically, the plates were processed to detect fluorescence intensity (Fluoro-Units: FU) at an emission wavelength of 528 nm and excitation wavelength of 485 nm using a fluorescence reader, FLx800 (Bio-Tek Instruments, Vermont, USA), similar to a method used to detect measles neutralizing antibodies [19]. To evaluate the requirement of complement, various concentrations of guinea pig complement (Denka Seiken, Tokyo, Japan) were added to the neutralization mixture of serially

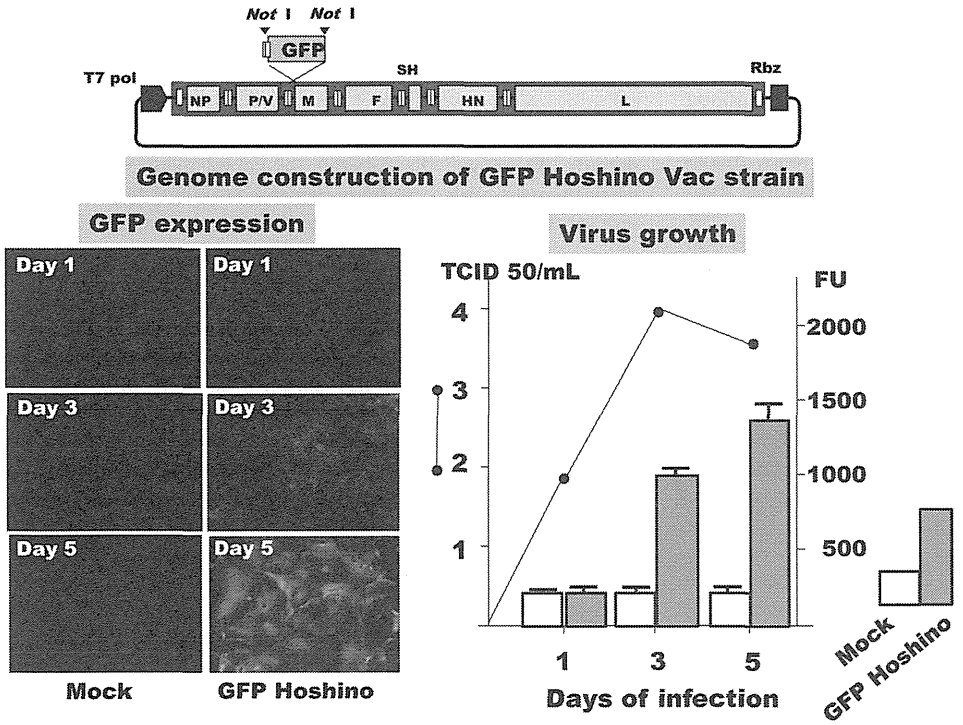

Figure 1. Genome construction of the recombinant mumps Hoshino strain expressing GFP and expression of GFP. Vero cells were infected with GFP Hoshino mumps strain at m.o.i. = 0.02 and subjected to experiments for GFP expression with fluoro EIA and microscopic examination on day 1, 3 and 5 of infection in comparison with mock-infection. Infectivity was assayed in culture supernatants on day 1, 3, and 5 of infection.
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diluted serum with challenge virus. Neutralizing antibody titers were determined as the reciprocal of the highest dilutions that did not exceed two-fold of FU values (GFP expression) of the cell control wells. Conventional neutralizing antibody titers were expressed as the reciprocal of the serum dilutions that showed 100% inhibition of CPE. Infectivity titer of the challenge virus was back-titrated in each assay, showing 50–120 $TCID_{50}$.

Statistical Analysis

Statistical significance in the neutralizing antibody titers was examined between two groups by the Mann-Whitney test. A coefficient was used for the analysis of correlation between the NT and EIA titers.

Results

GFP Expression

GFP expression and the viral growth are shown in Fig. 1. Vero cells were infected with the GFP Hoshino strain in a 24-well plate, and culture fluids were obtained 1, 3, and 5 days later. A peak infective titer of 10^4 TCID 50/ml was obtained 3 days after infection. Mean GFP expression (FU) is shown with 1.0 standard deviation (SD) in four wells in comparison with mock-infected wells. Mock-infected wells showed approximately 300 FU during the experiment, and GFP expression in infected wells increased to peak (1,300 FU) on day 5 of infection. Together with FU in fluoro-ELISA, fluoro-microscopic findings of CPE expansion with GFP expression are also shown in Fig. 1. Few CPE were observed on day 3 of infection and extensive cell fusion was noted on day 5. The development of CPE was closely related to GFP expression.

Neutralizing Antibody Titers

The results of the neutralization tests are shown in Fig. 2. Serum samples were serially diluted 2-fold from 1:4 to 1:256, and mixed with the challenge virus. The NT assay was done in duplicate. The results for one serum sample are shown in Fig. 2. CPE were observed in one well at 1:32 and none at 1:16. The conventional neutralizing antibody titer was considered to be 1:16 for 100% inhibition of CPE. The mean FU of cell control wells (mock-infected wells) was 202 FU. The mean FU of serial dilutions from 1:4 to 1:256 was 252 FU, 239 FU, 234 FU, 450 FU, 543 FU, 581 FU, and 591 FU, respectively. GFP expression increased to 450 FU at 1:32 and thus the neutralizing antibody titer for the GFP expression assay was 1:16 for inhibition of the growth of GFP Hoshino. The infective titers of the challenge virus were back-titrated, showing 50–120 TCID 50. When CPE appeared in >20% of the wells, GFP expression was >500 FU.

To evaluate the consistency of neutralizing antibody titers assayed by 100% inhibition of the appearance of CPE or GFP expression, neutralization tests for both conventional and GFP expression methods were done in 1,452 fresh serum samples. Three cut-off levels for positive GFP expression were set: 1.5-, 2.0-, and 2.5-fold increase in FU compared to cell culture controls. Among the 1,452 samples, 1,287 (88.6%) showed the similar neutralizing antibody titers when assayed by both methods using the 1.5-fold cut-off, 1,367 (94.1%) with the 2.0-fold cut-off, and 1,058 (72.9%) with the 2.5-fold cut-off. Strong similarity was noted when the cut-off was defined as a 2.0-fold of FU value in FU of the control wells.

Effect of Heat Inactivation and Addition of Complement

Eight fresh serum samples (A–H) were obtained and stocked at −80°C. Neutralizing antibody titers were examined before freeze-thawing, and after three and five rounds of freeze-thawing. The results are shown in Fig. 3. For serum A, the neutralizing antibody titer was 1:256, 1:64, and 1:128, showing no significant difference within five rounds of freeze-thawing. It decreased to 1:8 or 1:16 after inactivation at 56°C for 30 min. The other serum showed similar results. Neutralizing antibody titers did not decrease but decreased after inactivation of the complement. Complement activity would be required for neutralization tests for mumps virus.

Five fresh serum samples (A–E) were inactivated at 56°C for 30 min. When inactivated sera were used, guinea pig complement was added to the neutralizing mixture at 1:200, 1:400, 1:800, and 1:1,600. Neutralizing antibody titers were examined and mean values for three independent assays are shown in Fig. 4. Guinea pig complement did not affect the assay system without any changes in Vero cell cultures and the addition of guinea pig complement in non-inactivated sera did not influence the neutralizing antibody titers. The titer was 1:32–1:128 and dropped to around 1:8 after inactivation. The reduced neutralizing antibody titers increased to levels similar to those before inactivation when the complement was added at 1:200 or 1:400. Therefore, complement was added at 1:200 to the neutralizing mixture in the subsequent experiments.

Effect of Complement

Twenty-one fresh serum samples were obtained and neutralizing antibody titers were examined for non-inactivated and inactivated sera supplemented with complement at 1:200 in the neutralizing mixture. The results are shown in Fig. 5. The peak distribution of neutralizing antibody titers for non-inactivated samples was 1:32 and shifted to 1:64, showing no significant change in those with addition of complement.

As for the 227 stocked sera, neutralization tests were performed before and after inactivation with the addition of complement. 74 serum samples showed negative and 70 became positive, when assayed after inactivation with the addition of complement. The peak distribution of neutralizing antibody titers markedly shifted from 1:4 for non-inactivated sera (98 sera) to 1:16 after inactivation supplemented with complement (75 sera). Stocked sera were considered to lose complement activity over long periods. Therefore, the addition of complement was required when the neutralizing antibody titer was examined for the stocked sera, probably because of decreased complement activity.

Discussion

There are several serological methods of detecting mumps antibodies. Complement fixation (CF) and hemagglutination inhibition (HI) tests are not sensitive and, in addition, HI antibodies are cross-reactive to parainfluenza virus [1,10]. EIA has high sensitivity and specificity and is a simple procedure, but is not related to protective activity [11]. Neutralizing antibodies are associated with protective activity but the neutralizing test involves several complicated steps. Neutralization of an infectious virus and the preparation of cell cultures are bothersome and most time-consuming is the very last step to determine the appearance of CPE in 96-well plates. For micro-neutralization assays, there are two methods; 50% plaque reduction and complete inhibition of CPE. There have been several reports on neutralizing tests, concerning the evaluation of plaque reduction or inhibition of the appearance of CPE, infectivity of a challenge virus, and requirement of complement for neutralizing tests [12,13,14,15].

Fujino et al. [19] reported the neutralization test for measles virus using a GFP-expressing recombinant measles virus to evaluate the neutralizing antibody titer by Fluorescent EIA reader. Here, a recombinant mumps Hoshino vaccine strain expressing

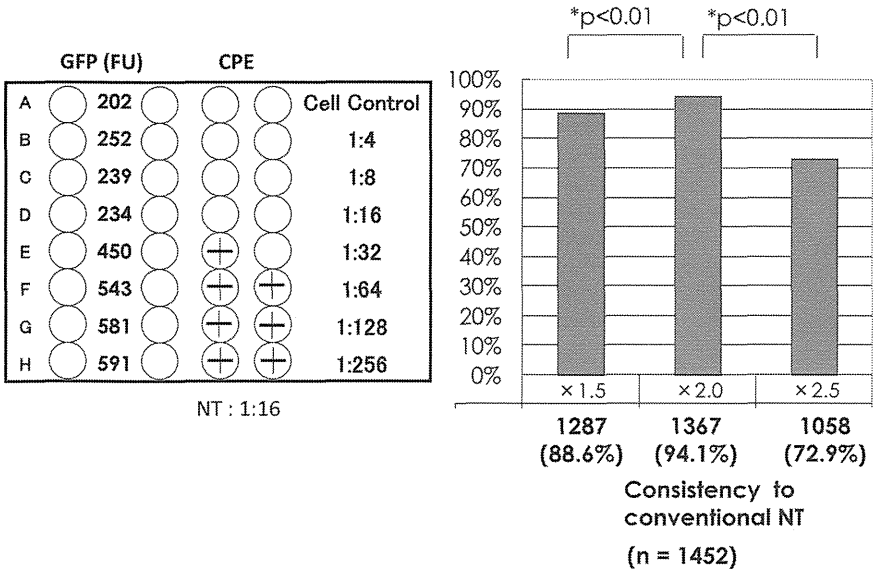

Figure 2. Relationship between the appearance of CPE and GFP expression. Serial two-fold dilutions from 1:4 to 1:256 were mixed with an equal volume of challenge virus. In the left panel, the schematic results of two neutralization methods are shown. CPE was observed in one of the two wells at 1:32, and the conventional neutralizing antibody titer was 1:16 by 100% inhibition of CPE. The mean FU value of the two cell control wells was 202 and that of the 1:32 dilution was 450, showing 1:16 of neutralizing antibody titer. Using 1,452 serum samples, the consistency of neutralizing antibody titers was compared based on different cut-off values for GFP expression: 1.5-fold, 2.0-fold, and 2.5- fold of FU values of the cell control wells.
doi:10.1371/journal.pone.0065281.g002

GFP was developed to check the expression of GFP instead of observing the appearance of CPE or plaque counting. GFP expression was examined by a fluorescent EIA reader as fluoro-units (FU). GFP expression increased as the virus genome was

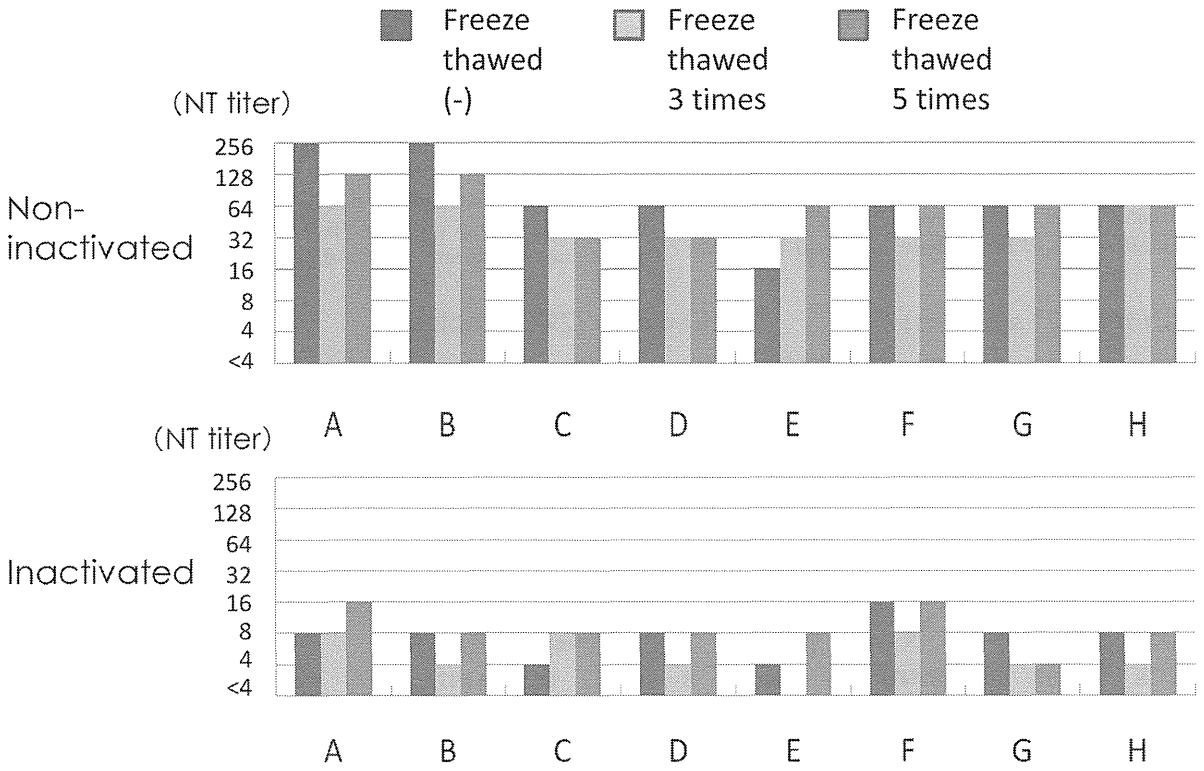

Figure 3. Effects of freeze-thawing and inactivation at 56°C for 30 min on neutralizing antibody titers. Upper panel shows the neutralizing antibody titers of eight fresh sera (A–H), without inactivation and after three or five rounds of freeze-thawing. Lower panel shows the results of neutralizing antibody titers after inactivation.
doi:10.1371/journal.pone.0065281.g003

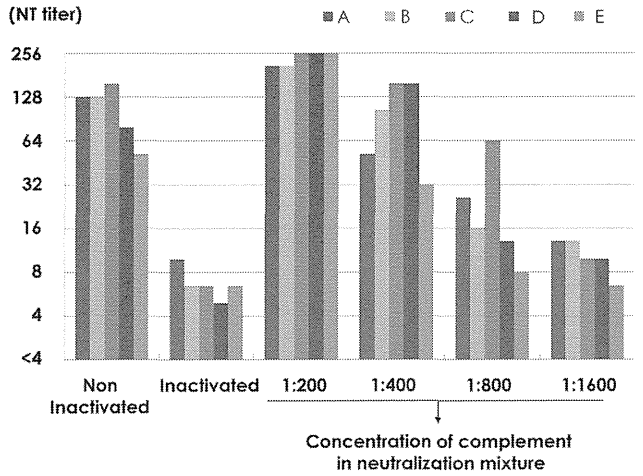

Figure 4. Neutralizing antibody titers of non-inactivated and inactivated sera with the addition of complement. Neutralizing antibody titers were examined in five sera (A–E) before and after inactivation. Complement was added at 1:200, 1:400, 1:800, and 1:1600 to the neutralizing mixture when inactivated sera were used. Each experiment was done in triplicate and mean titers were shown.
doi:10.1371/journal.pone.0065281.g004

transcribed after infection and was closely related to viral growth, as shown in Fig. 1. GFP expression in the cell control wells in a 96-well plate was approximately 200 FU. More than a two-fold increase in FU was considered positive for GFP expression (presence of CPE). Neutralizing antibody titers examined by GFP expression were similar to those by the conventional method for 100% inhibition of CPE (Fig. 2).

In several reports, the neutralizing step was performed without the addition of complement. Hishiyama et al. [15] reported that fresh guinea pig serum was required for neutralization tests for mumps virus. They used complement at 1:400 dilutions in the neutralizing mixture and the addition of complement increased the neutralizing antibodies titers. Complement has several important roles in immune responses and there are three main pathways, the classical, lectin, and alternative pathways. Complement is one of the first lines of host defense and is an important part of humoral immune responses. The complement system is immediately ready to target and eliminate viral particles and to interact with specific antibodies on the surface of a virus or infected cells [20]. Complement-dependent neutralizing antibody is reported to recognize the viral glycoproteins on the virus envelope, directly related to neutralization of Vesicular stomatitis virus [21,22], herpes simplex viruses [23,24], and West Nile virus [25]. Cooper et al. [26] reported that the deposition of antibody and complement on the surfaces of viral particles might physically interfere with infectivity in susceptible cells due to aggregation of the viral particles. However, Friedman et al. [23] suggested that complement inhibited the infection process of HSV, indicating that it affects viral replication: virus entry, uncoating, DNA transport to the nucleus, or immediate early gene expression, not requiring particle aggregation, viral lysis, or blocking of virus attachment. Johnson et al. [16] investigated the requirement of a complement system to neutralize three closely related paramyxoviruses, Simian virus 5 (SV5), mumps virus, and human parainfluenza virus type 2 (HPIV2). HPIV2 was neutralized in a complement-independent manner but neutralization of SV5 and mumps virus proceeded through alternative pathways. However, they were neutralized by different mechanisms; C3 deposition was observed on the surface of SV5 particles, resulting in aggregates. C3 deposition was also noted on the surface of mumps virus particles but they induced virion lysis through electron microscopic findings. In this sense, the presence of complement seemed to be essential for the neutralization tests for mumps virus. When fresh sera were examined for the detection of neutralizing antibodies against mumps virus, the addition of complement did not enhance the neutralizing antibody titers and the titers were stable for 5 rounds of freeze-thawing. But the complement activity was reduced after inactivation and during long-term preservation, and the addition of complement at 1:200 was required for neutralization tests against mumps virus.

EIA is simple and a large number of serum samples are handled without serial dilutions, which is suitable for surveillance but does not reflect protective immunity. A purified mumps virus antigen is used for the EIA antigen, and contains component proteins as well as viral particles. In our previous report, neutralizing antibodies assayed by the conventional method without complement showed a poor relationship to EIA titers. In the present study, there was again no significant relationship, with a low co-efficiency,

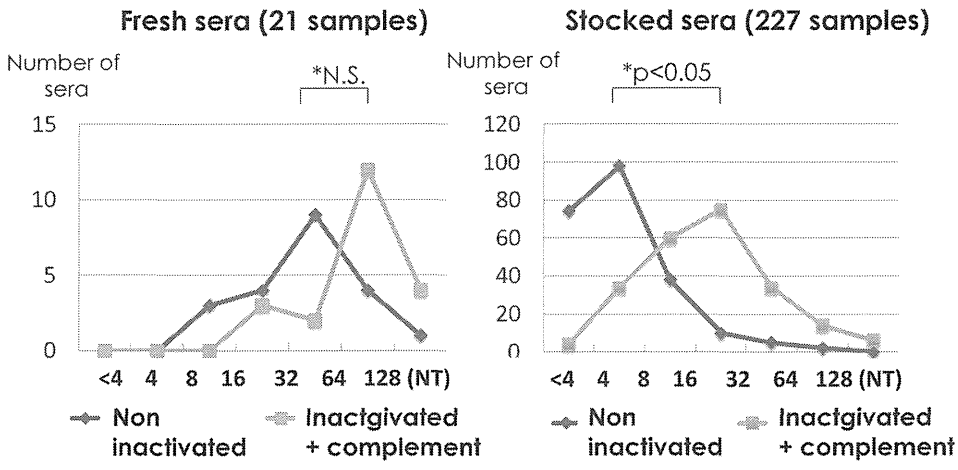

Figure 5. Effect of the addition of complement in 21 fresh and 227 stocked serum samples. Distribution of serum samples is shown for neutralizing antibody titers assayed without inactivation and for those assayed after inactivation with the addition of complement, using 21fresh serum samples (left panel). 227 stocked serum samples were assayed in a similar manner (right panel).
doi:10.1371/journal.pone.0065281.g005

examined by adding complement (data not shown). EIA-positive sera showed positive immune-fluorescent antibodies against the most abundant N protein [27]. Approximately 40–50% of the serum samples positive for neutralization test showed positive for immune-fluorescent antibodies against F or HN antigens, which are closely related to the infection process, attachment and cell fusion [27].

Using a recombinant mumps virus expressing GFP, the neutralization test was simplified via a reduction in GFP expression, counting automatically by fluorescent EIA reader. When stocked samples were used, complement was added at a concentration of 1:200.

Author Contributions

Conceived and designed the experiments: TN. Performed the experiments: KM MF. Analyzed the data: KM TN. Contributed reagents/materials/analysis tools: MF KT SI. Wrote the paper: KM TN.

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REVIEW ARTICLE

Vaccine chronicle in Japan

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Abstract The concept of immunization was started in Japan in 1849 when Jenner's cowpox vaccine seed was introduced, and the current immunization law was stipulated in 1948. There have been two turning points for amendments to the immunization law: the compensation remedy for vaccine-associated adverse events in 1976, and the concept of private vaccination in 1994. In 1992, the regional Court of Tokyo, not the Supreme Court, decided the governmental responsibility on vaccine-associated adverse events, which caused the stagnation of vaccine development. In 2010, many universal vaccines became available as the recommended vaccines, but several vaccines, including mumps, zoster, hepatitis B, and rota vaccines, are still voluntary vaccines, not universal routine applications. In this report, immunization strategies and vaccine development are reviewed for each vaccine item and future vaccine concerns are discussed.

Keywords Vaccine · Surveillance · MMR · DPT · Voluntary vaccines · Recommended vaccines

Abbreviations

ACIP	Advisory Committee on Immunization Practices
BCG	Bacillus Calmette–Guérin
DTaP	Acellular pertussis vaccine combined with diphtheria and tetanus toxoids
DTwP	Whole cell pertussis vaccine combined with diphtheria and tetanus toxoids
FHA	Filamentous hemagglutinin
HA	Hemagglutinin
HBV	Hepatitis B virus vaccine
Hib	*Haemophilus influenzae* type b conjugated with tetanus toxoid
HPV	Human papilloma virus vaccine
JEV	Japanese encephalitis vaccine
IPV	Inactivated polio vaccine
LAMP	Loop-mediated isothermal amplification
MMR	Measles, mumps, and rubella-combined vaccine
MR	Measles and rubella-combined vaccine
NA	Neuraminidase
NT	Neutralization test
OPV	Live oral polio vaccine
PCV7	7-valent *Streptococcus pneumoniae* conjugated vaccine with recombinant diphtheria toxoid
PT	Pertussis toxin
Tdap	Tetanus toxoid combined with a reduced concentration of diphtheria toxoid and acellular pertussis
VAP	Vaccine-associated paralytic polio
VZV	Varicella zoster virus vaccine

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Dawn of vaccines in Japan

The dawn of vaccinology was the first scientific systematic investigation of the cowpox vaccination by Edward Jenner in 1796, although several variations in approach were performed using dried pus from smallpox skin lesions in Central Asia, China, and Turkey [1]. Jenner's cowpox vaccination procedure was introduced into Japan in the Edo era by Philipp F.B. von Siebold. Sporadic nationwide outbreaks occurred at that time, which caused social, economic, and political stagnation, and doctors of herbal traditional medicine, studying Western modern medicine, wanted to use Jenner's cowpox vaccine as a preventive

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procedure for smallpox. Many attempts were made to import the cowpox seed, but these did not succeed because Jenner's cowpox vaccine is a live vaccine: it was inactivated during long-term transportation or if the inoculation chain in children was interrupted. It was finally introduced to Nagasaki in 1849, bringing the vaccination scar through the idea proposed by Dr. Souken Narabayashi, who was the chief doctor of Nabeshima-Han (Saga Prefecture). The vaccination procedure became available at the Shutousyo (Vaccination Institute) in Osaka and Edo cities, which was the origin of the School of Medicine of Osaka and Tokyo Universities [2]. Jenner's cowpox vaccine gained in popularity because of its distinct effectiveness against smallpox. However, some opinions were against vaccination because of misunderstanding involving unreasonable and nonscientific rumors, as has recently been observed.

The Japanese government in the Meiji era decided that all Japanese people should be immunized with the vaccine for smallpox, which was stipulated in 1876, and a vaccination law against smallpox started in 1910. The present immunization law was implemented in 1948 under occupation by the United States (US). Issues related to vaccine development and immunization policies are summarized in Table 1. There have been two turning points for amendments to the immunization law: the compensation remedy for vaccine-associated adverse events in 1976, and the concept of private vaccination in 1994. These two turning points were attributed to vaccine-associated adverse events or accidents and lawsuits against serious neurological adverse events after immunization with vaccinia and the measles, mumps, and rubella-combined vaccine (MMR) [3]. In 1992, the regional Court of Tokyo, not the Supreme Court, set the governmental responsibility for vaccine-associated adverse events because the government did not make an effort to enlighten the public and doctors by explaining the possible adverse events associated with vaccinations, even though immunization was recommended to be compulsory [3]. This lack of information was a major reason why the government was reluctant to take active immunization strategic action, leading to the so-called long-term vaccine gap after the discontinuation, in 1993, of MMR, which had been introduced in 1989, because of the unexpectedly high incidence of aseptic meningitis caused by mumps vaccine components [4, 5]. The mechanisms behind the higher incidence of aseptic meningitis with the combined live MMR vaccine than with monovalent mumps vaccines were not clearly identified. Thereafter, new vaccines were not introduced, but many pediatric vaccines have been approved in developed countries, with the implementation of recommended vaccines, which shows that vaccine-preventable diseases should be controlled with available vaccines

Table 1 History of immunization and vaccine development in Japan

1948: Immunization Law [Smallpox, Diphtheria, Typhoid fever, Salmonella Paratyphi, Pertussis, Tuberculosis, Typhus, Plague, Cholera, Scarlet fever, Influenza, Leptospirosis]	
1951: Preventive law against tuberculosis.	
1961: The polio vaccine was recommended.	
1962: School immunization with the influenza vaccine	Adverse events after the smallpox vaccination 1968–1970
1968: DPwT was recommended vaccination 1968–1970	
1976: Amendment of the immunization law for a compensation remedy for adverse events: Recommended obligatory [Smallpox, Diphtheria, Tetanus, Pertussis, Polio]; Temporarily [influenza, JEV]	DPT accidents 1974–1975
1977: The rubella vaccine was recommended.	
1978: The measles vaccine was recommended.	
1980: Eradication of smallpox and stopped being used.	
1981: The mumps vaccine was licensed.	MMR scandal 1989–1993
1985: The hepatitis B vaccine was licensed for the prevention of vertical transmission in1986.	
1994: Ammendment for private vaccination: Recommended [DPT, Polio, Measles, Rubella, JEV] Voluntary [influenza, VZV, Mumps]	
1995: The hepatitis A vaccine was licensed,	
2001: The influenza vaccine was recommended for the elderly >65 years.	
2005: BCG was recommended for infants 0–6 months of age.	JEV ADEM 2005
2005: The JEV vaccination was interrupted until 2009 and a booster at 14 years was stopped.	
2006: The two-dose schedule was started, using the MR combined vaccine.	
2009: Pandemic 2009 vaccines were imported from GSK and Novartis.	
2010: Hib, PCV7, and HPV were temporarily recommended.	

DPwP Whole cell pertussis vaccine combined with diphtheria and tetanus toxoids, *JEV* Japanese encephalitis virus vaccine, *MMR* Measles, mumps and rubella-combined vaccine, *VZV* Varicella zoster virus vaccine, *ADEM* Acute disseminated encephalomyelitis, *Hib* Haemophilus influenzae type b vaccine, *PCV7* 7-valent Str. pneumoniae vaccine, *HPV* Human Papilloma virus vaccine

[6–9]. *Haemophilus influenzae* type b conjugated with tetanus toxoid (Hib) was introduced in 2008, and 7-valent *Streptococcus pneumoniae* conjugated vaccine with recombinant diphtheria toxoid (PCV7) and human papilloma virus vaccines (HPV) became available in 2010. Rotavirus vaccines were introduced in 2012. Several issues concerning vaccines in Japan are discussed in this article.

Immunization law and schedule

The Japanese immunization law is complicated with double-standard categories: routine recommended and voluntary vaccination. Routine recommended vaccines consist of BCG, acellular pertussis vaccine (DTaP), measles and rubella combined vaccine (MR), inactivated polio (IPV), Hib, PCV7, HPV, and Japanese encephalitis vaccine (JEV). Voluntary vaccines are hepatitis B (HBV), mumps, varicella, and rotavirus vaccines. The difference between the two is the cost of immunization; routine recommended vaccines are principally covered by the regional government [10, 11]. Until 1994, immunization was performed by mass vaccination in regional Public Health Centers. It was replaced by private vaccination, derived from the concept that it is better that vaccinations are performed by children's family doctors who are familiar with their health conditions. Although this concept was easily accepted by general physicians, mass vaccination of BCG still continued in some regions.

In 2010, Hib, PCV7, and HPV began to be used as temporarily recommended vaccines, and the cost was partially supported by the regional governments [12]. Vaccination coverage of routine recommended vaccines is more than 90–95 % for BCG, DTaP, OPV, and MR and 80 % for JEV, whereas that of voluntary vaccines is less than 30–40 %. During 1990–2000 polyvalent combined vaccines were developed in the EU and widely used. There is no licensed polyvalent vaccine in Japan, and the vaccination schedule became much tighter than that in the 1990s, especially in very young infants less than 6 months of age (Fig. 1). Simultaneous administration of several vaccines was recommended by the Japanese Pediatric Association, as has been conducted in the US and EU [3, 4]. In March 2011, seven infants died within a week of receiving DTaP, Hib, PCV7, or BCG. The newly introduced Hib and PCV7 were temporally discontinued, but were restarted 1 month later because the risk of serious adverse events was not higher than that reported in developed countries. Simultaneous administration has been safely and effectively performed in the US and EU; however, the incidence of serious adverse events has been reported as 0.02–1 in 100,000 [13]. Therefore, simultaneous immunization is now performed without a high level of confidence. Careful surveillance monitoring and

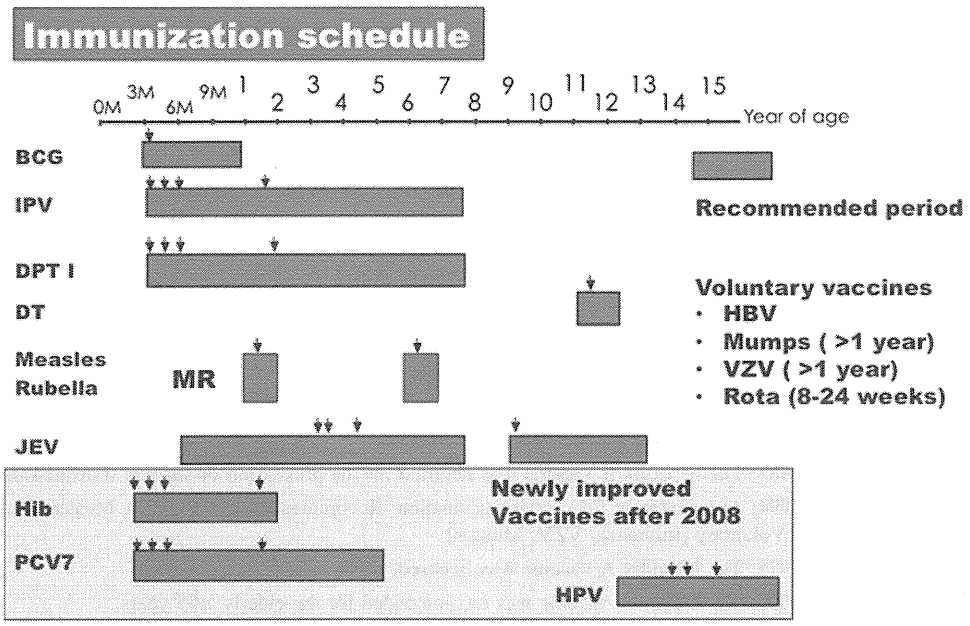

Fig. 1 Immunization schedule. BCG, IPV, DPT, DT, MR, JEV, Hib, PCV7, and HPV were recommended vaccines and HBV, Mumps, VZV, and Rota vaccines were voluntary vaccines. *Arrows* show the recommended timing for vaccinations. *BCG* Bacillus Calmette Guérin, *IPV* Inactivated polio vaccine, *DPT* Diphtheria and tetanus toxoids combined with pertussis vaccine, *DT* Diphtheria and tetanus toxoids, *MR* Measles and rubella-combined vaccine, *JEV* Japanese encephalitis vaccine, *Hib* Haemophilus influenzae type b vaccine, *PCV7* 7-valent Str. pneumoniae vaccine, *HPV* Human papilloma virus vaccine, *HBV* Hepatitis B virus vaccine, *VZV* Varicella zoster virus vaccine

scientific investigations are required to define the safety of simultaneous immunization.

Measles and rubella elimination

In Japan, live attenuated measles vaccines were developed in the 1970s, and four strains were licensed (three strains are used at present) with the implementation of recommended immunization in 1978 [14]. Five strains of live attenuated rubella vaccines (three strains are used at present) were developed and recommended for female students aged 14 years in 1977 [15]. Surveillance data and changes in the vaccination policy against measles and rubella are shown in Fig. 2. The MMR vaccine was used between 1989 and 1993 but was discontinued in 1993. Measles and rubella monovalent vaccines have been used for children aged 12–90 months since 1994 to control measles and rubella because the number of patients with congenital rubella syndrome did not decrease as a result of the vaccination of only young females since 1977.

Regarding the reporting system for measles in Japan, through 3,000 sentinel clinics or hospitals for pediatric infectious diseases and 450 clinics for adult measles surveillance, patients with clinically suspected measles were reported to Regional Health Care Centers. In the late 1990s to early 2000s, 20,000–30,000 cases of measles, including several dozen deaths, were reported yearly. A total of 2,034 cases of

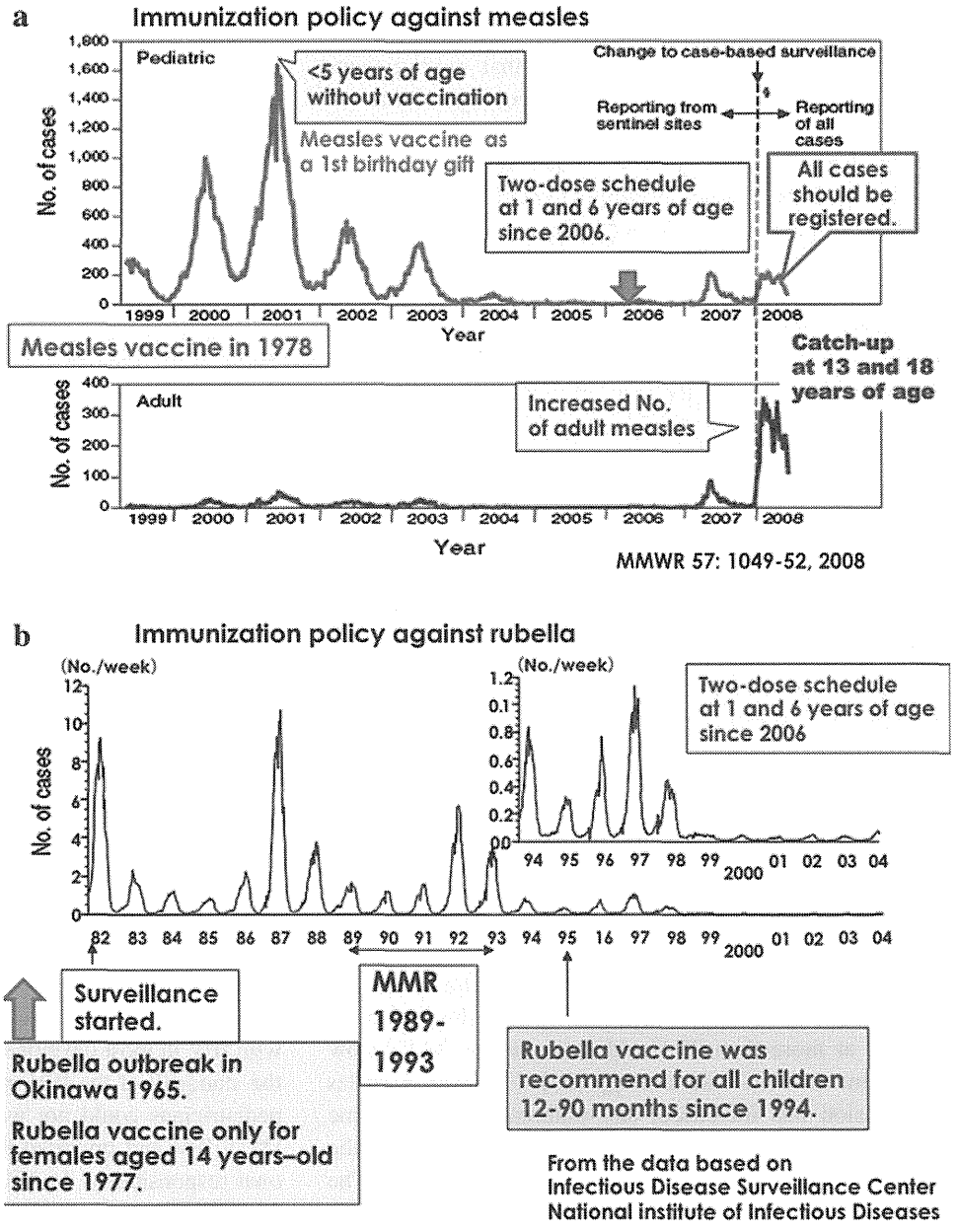

Fig. 2 Surveillance results of measles (**a**) and rubella (**b**), and the changes in immunization policies. Measles and rubella vaccines were recommended in 1978 and 1977, respectively. The MMR vaccine was used between 1989 and 1993, and the target generation of the rubella vaccine shifted from 14-year-old female schoolchildren to all infants 12–90 months of age. The two-dose schedule of the MR combined vaccine started in 2006. A catch-up campaign started in 2008 for an additional 5-year schedule for children 13 and 18 years of age. *MMR* measles, mumps, and rubella-combined vaccine

measles, including 8 deaths, were reported in a severe measles outbreak in Okinawa in 1998–1999 [16]. Many of the deaths occurred in infants under 1 year of age. A large measles outbreak was observed in 2001 in Japan. Among 33,812 reported cases, most patients were under 5 years of age and had not been vaccinated. Through a vaccination campaign to increase immunization coverage at 1 year of age, the number of reported cases decreased to 545 in 2005. The Japanese Government implemented a two-dose strategy in 2006, a combined measles and rubella vaccine (MR) for children at 1 and 6 years of age [17]. Therefore, the elimination of measles was expected. However, patients with measles were increasingly reported in March 2007, and this outbreak subsequently expanded throughout the Japanese districts, peaking in the middle of May. Furthermore, several reports indicated measles transmission by Japanese travelers or participants in an international sporting event [18–20]. This outbreak showed different characteristics, demonstrating that most patients were young adults or adolescents attending high school and university students, with a much lower proportion of young infants, at the early stage of the outbreak [21]. Cases of measles were reported in all age populations, with a total of 3,105 pediatric cases and 959 adult patients being reported in 2007. The number of patients with measles was the highest between 1 and 4 years of age, accounting for 40–50 % in 2001, which decreased to 22 % in the outbreak of 2007. A significant shift in the age distribution of cases of measles in 2007 was observed to be 10–14 years or older, accounting for 44 % in 2007 [22].

To reduce the number of patients with measles, an additional MR catch-up campaign was started for teenagers at the age of 13 and 18 years (MR III and IV) from 2008 for a 5-year schedule. After 2008, all cases with measles had to be registered, and the number of patients with measles was reduced to 457 cases in 2010 (3.58 cases per million), with some imported genotypes [23]. In 2011, measles was introduced from the EU by a journalist who was collecting the news of the earthquake, tsunami, and nuclear power disaster, and a total of 442 patients with measles were finally reported [24]. In 2012, 293 patients were reported, just on the edge of measles elimination of 1 case in 1,000,000, and most cases were identified as importations from Southeast Asia and the EU [25].

Global measles vaccination coverage increased from 72 % in 2000 to 82 % for the first dose in 2007, and the two-dose immunization strategy was recommended for countries with high coverage of the first-dose measles vaccine, at more than 95 %. Most countries (88 %) now implement the two-dose strategy [26]. However, measles transmission has increased, and outbreaks have become widespread since late 2009 in the EU region because of the failure to immunize susceptible populations [24]. The World Health Assembly updated the goal of measles elimination to a 95 % reduction in measles mortality by 2015, compared to 2000 [27].

The rubella vaccine strategy was markedly changed in 1994. Before 1989, the rubella vaccine was administered to 14-year-old girls, but the vaccine target has changed to all children aged 12–90 months. Boys more than 90 months of age and girls from 90 months to 14 years of age were not enrolled as immunization targets in the transition period. Even though a temporal catch-up campaign was conducted to cover the immunization gap, vaccine coverage was extremely low [15, 28]. According to the immunization gap in younger generations around 30 years of age, an outbreak of rubella was observed in 2011–2013, with some imported cases from Southeast Asia, resulting in congenital rubella syndrome [29]. Rubella is now prevalent (in 2013) among men around 30 years of age who have not been immunized because of the immunization gap. Through the enhanced network activity of measles and rubella elimination, the elimination of rubella has been targeted in accordance with measles elimination, using the measles and rubella combined vaccine [30, 31].

Replacement of oral polio vaccine (OPV) with inactivated polio vaccine (IPV)

Surveillance data of reported cases of polio paralysis are shown in Fig. 3. In 1960, a nationwide outbreak was observed, and approximately 5,800 patients with paralytic polio were reported. A similar level of outbreak seemed to be observed in 1961, and the Japanese government decided to import sufficient doses of OPV for all Japanese children. Within a month, 15 million doses were given to all Japanese children less than 5 years old. Around 1960, although IPV was under investigation and a clinical trial of imported OPV was also underway in Japan, the importation of OPV was politically decided. After the introduction of OPV in 1961 and afterward, the number of polio cases decreased [32]. After 1980, no wild strain was isolated from patients suspected of flaccid paralytic polio. All cases of paralytic polio were identified as vaccine-associated paralytic polio (VAP). The incidence of VAP was recently shown to be one in 1.4 million recipients in Japan. Clinical trials of domestic IPV produced from Sabin's live oral polio vaccine strains were performed beginning in 1998, but the application was withdrawn. Considering the practical way of immunization, the development of IPV combined with DTaP was more desirable than IPV alone. OPV was replaced with IPV in most developed countries, but it was delayed by the standstill of the IPV trial in Japan. Some guardians and pediatricians could not wait for the licensure of domestic DTaP/IPV and imported the IPV vaccine privately at their own responsibility. In 2012, IPV was allowed for use as a recommended vaccine imported from Sanofi and domestic