Latex Particles Coated With Sialic Acid-Containing Glycoprotein Are Agglutinated By Influenza Viruses

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Introduction

Influenza virus is a cause of respiratory illness which is a major public health problem worldwide. Although the term "influenza pandemics" has greater impact, over the long term the smaller influenza epidemics that occur each winter between "global pandemics" are responsible for greater overall mortality. To give immunization to large human population is the best protection against influenza outbreaks. Each year a new influenza vaccine is produced based on WHO recommendations. Therefore, prediction of the viral strains which will spread in the next season is critical for recommendations. Antigenic differences between isolates can be detected using ferret antisera and RBCs for analysis of cross reactivity in HI tests. However, the titers of HA tests depend on the species of RBCs used for the tests. In this study, we made the artificial particles which are able to be preserved for a long time, and examined whether they were able to be used instead of RBCs for HA and HI test.

Materials and Methods

Viruses. Influenza viruses used in this study were isolated from clinical specimens using MDCK cells or from avian feces using embryonated hen's eggs of our institute according to standard procedure. These isolates were then propagated using the same method as for isolation and stored at -80°C until use. We also used the inactivated strains for reference antigens provided by the National Institute of Infectious Diseases, Tokyo, Japan and A/turkey/Ontario/7732/66(H5N9) and A/duck/Hongkong/342/78(H5N2) avian influenza live strains stocked and propagated in our institute.

Buffers and reagents. LSTc and BSA (98-99%, fraction V) were obtained from Sigma-Aldrich Co, St. Louis, MO, USA. LSTa was obtained from Dextra laboratories, Ltd, Whiteknights Road, Reading, UK. MES, NHS, EDAC were also obtained from Sigma-Aldrich Co. CML particles were obtained from Polysciences, Inc,Warrington, PA, UK.

Sialylated glycoconjugates. The synthesis of the sialic acid containing glycoconjugates was according to the literature [1]. Briefly, 10.4 mg of LSTc and 10.7 mg of bovine serum albumin (98-99%, fraction V) were dissolved in 1.5 ml of 0.2M borate buffer pH8.7 [2]. The solution was stirred for 1h at 55°C, then 26 mg of $NaBH_3CN$(WAKO Chemical Co, Japan) was added, and the mixture was stirred for 24 hours at 55°C. The solution was dialyzed against distilled water and lyophilized to give 13.2mg of a white powder. The same procedure was applied for the preparation of linking with LSTa and BSA.

Coupling glycoprotein to microparticles. Covalent coupling procedure of glycoprotein to micro particles was guided by the maker's instruction book. In detail, 160μl solution of 2.5%CML particles with 1.0μm diameter, 40μl of 500mM MES buffer, 92μl of 50mg/ml NHS, 16μl of 10mg/ml EDAC and 92μl of distilled water were pipetted into a microcentrifuge tube, and incubated at room temperature on a mixing wheel for 30 minutes. The tube was centrifuged and supernatant was discarded, then particles were resuspended with 1ml of 50mM MES buffer, pH6.1. After repetition of centrifugation and removal of supernatant, the pellet was resuspended with 20μl of 500mM MES buffer, 80μl of glycoprotein stock (1mg/ml of 0.3M sodium phosphate buffer pH 8.7) and 100μl of DW. The mixture was incubated at room temperature on a mixing wheel for 1 hour. The tube was centrifuged and microparticles ware washed 2 times with 50mM MES to remove free glycoprotein. The glycoprotein coupled microparticles were suspended in the 0.1M glycine-NaOH buffer (pH 8.1) containing 0.14M sodium chloride and 0.1% BSA to make a final concentration of 0.05%.

HA, HI assay and PA, PI assay. All of the HA, HI assay and PA, PI assays were performed using 96-well microtiter plates with V-shape bottoms. The HA titration and the HI test were guided by the recommended protocol of WHO collaborating centre for influenza. The PA and PI assay were performed in the same way as the HA and HI. Erythrocyte suspension obtained from human (blood type O) or chicken were stored in Alsever's reagent at 4°C until use. 0.7% human erythrocytes suspension or 0.5% chicken erythrocytes suspension was used for the HA and the HI assay. The reactions of the HA and the PA titration were done at 4°C for 16 hours. Ferret post-infection antisera panels against each reference antigen were also provided by the National Institute of Infectious Diseases, Tokyo, Japan. Ferret antisera were ware reconstituted and treated with RDE at 37°C over night. The next day, the sera were heated at 56°C for 60 minutes and treated with packed RBC to remove of nonspecific agglutinins.

Neuraminidase treatment of microparticles. -2-(3,6,8,9)-Neuraminidase from *Arthrobacter ureafaciens* and reaction buffer were obtained from Sigma. Total 0.1ml of reaction mixture containing 4% (V/V) of washed LSTc-BSA-CML in reaction buffer with 2.5mIU of neuraminidase was incubated at 37°C for 3 hours. Neuraminidase treated particles were washed with 50mM MES buffer and pelleted by centrifugation. The microparticles were suspended in the 0.1M glycine-NaOH buffer (pH8.1) containing 0.14M sodium chloride and 0.1% BSA to make a final concentration of 0.05%.

Reading of the plates. Confirmation of the PA results was according to the previous report [3]. Briefly, the results were obtained by inclining the plates for 30 seconds. At the end of this time, if a layer or button of latex on the well bottom did not flow and no line was seen, it was judged as positive. If a discreet button of latex flowed and a line was seen, it was judged as negative.

Results

Comparison between HA and PA titers. The latex particles linking with LSTc-BSA showed comparable agglutination titers to HA titers when using H1 subtype, most of H3 subtype and B type influenza viruses. In contrast, the PA result of two H3 subtype isolates named A/Osaka/01/2004 and A/Osaka/18/2005 was very low (at<8). (Table 1) As the negative control, BSA-linked latex were not agglutinated by any influenza viruses. The latex particles linking with LSTc-BSA after the treatment by α2-(3,6,8,9) neuraminidase at 37°C for 3 hours were not agglutinated by existence of any reference antigen. (data not shown).

Table 1. Comparison between the HA titers and the particles coated with LSTc-BSA agglutination titers used filed isolates.

Strain	HA titer*1	PA titer*2
A/Moscow/13/98(H1N1)	16	16
A/New Caledonia/20/99(H1N1)	64	64
A/Panama/2007/99(H3N2)	64	32
A/Wyoming/03/2003(H3N2)	64	64
B/Shandong/7/97(Victoria)	128	128
B/Johannesburg/5/99(Yamagata)	64	128
A/Osaka/374/2001(H1)	16	16
A/Osaka/585/2001(H1)	32	32
A/Osaka/868/2001(H1)	64	32
A/Kadoma/35/2002(H1)	128	32
A/Kadoma/51/2002(H1)	64	64
A/Kadoma/52/2002(H1)	64	64
A/Kadoma/54/2002(H1)	64	64
A/Osaka/01/2004(H3)	16	<8
A/Osaka/30/2004(H3)	16	8
A/Osaka/32/2004(H3)	64	16
A/Osaka/01/2005(H3)	16	8
A/Osaka/06/2005(H3)	128	64
A/Osaka/11/2005(H3)	128	128
A/Osaka/18/2005(H3)	64	<8
B/Osaka/01/2004(Yamagata)	32	32
B/Osaka/02/2004(Yamagata)	16	16
B/Osaka/03/2004(Yamagata)	32	64
B/Osaka/09/2005(Yamagata)	32	128
B/Osaka/10/2005(Yamagata)	64	64
B/Osaka/11/2005(Yamagata)	64	32
B/Osaka/13/2005(Yamagata)	32	64

*1 0.7% human typeO RBCs were used for hemagglutination assay
*2 0.05% particles were used for particle agglutination assay

Comparison between HI and PI titers. In case of A/Moscow/13/98 antiserum, the PI titers were ≤10 in all clinical isolates. In case of A/New Caledonia /20/99 antiserum, the PI titers were lower than HI titers when we tested the clinical isolates of H1 subtype. On the other hand, for type B influenza viruses, both HI and PI tests showed no cross-reactivity against B/Shandong/7/97 antiserum and very high titer against B/Johannesburg /5/99 antiserum. For H3 subtype isolates, the PI titers tend to be higher than HI titers against two distinct antiserum.

Comparison between HA and PA titers using avian influenza viruses. All of the avian influenza viruses agglutinated chicken erythrocytes as well as human erythrocytes. PA results using LSTa-BSA-CML show similar results to HA except for A/turkey/Ontario/7732/66 and A/duck/HongKong/342/78. (Table 2) Although their PA results for all avian viruses except the two mentioned above have shown slightly lower agglutination titer, using LSTc-BSA-CML, the titer remained within a 2-fold difference compared with LSTa-BSA-CML results. Only the A/turkey/Ontario/7732/66 virus showed very high PA titer, when used with LSTa-BSA-CML which has just Neu5Acα2-3Gal sequence. This result suggests A/turkey/Ontario/7732/66 virus recognizes strongly Neu5Acα2-3Gal sequence on the cell surface glycoprotein. On the other hand, avian influenza viruses can bind LSTc-BSA-latex which has only Neu5Acα2-6Gal sequence.

Table 2. Comparison between the HA titers and the PA titers using the same avian influenza strains.

Viral strain	Hemagglutination titres*1		Particle agglutination titres*2	
	Chicken RBC	Human-O RBC	LSTa-BSA-CML	LSTc-BSA-CML
A/teal/Osaka/1/2002(H10N1)	256	256	256	128
A/teal/Osaka/2/2002(H10N1)	>256	256	256	128
A/teal/Osaka/3/2002(H10N1)	>256	>256	>256	>256
A/teal/Osaka/4/2002(H10N7)	>256	256	128	128
A/teal/Osaka/5/2002(H10N7)	>256	>256	256	256
A/wigeon/Osaka/1/2002(H7N7)	128	128	64	32
A/turkey/Ontario/7732/66(H5N9)	256	256	≥1024	32
A/duck/Hongkong/342/78(H5N2)	64	64	128	16

Maximum dilution of virus capable of agglutinating red blood cells or particles coated with sialic acid-containing glycoprotein.

Discussion

In this study, we produced latex particles(called LSTc-BSA CML) that were agglutinated by influenza viruses and are stable over long periods. The particles could be used successfully in agglutination inhibition assay and for the most part the PA Titers were comparable to the HA titers.There are many sialyloligosaccharides of various structures and lengths on the RBCs surfaces. The difference in the titers between HI and PI in clinical isolates might be due to structural differences or density differences of the sugar chain on the surface between the RBCs and the particles. Regarding as the two H3 strains, A/Osaka/01/2004 and A/Osaka/18/2005, the results of HA were not parallel to PA. We tested agglutination titers of these two strain with chicken erythrocytes or latex particles linking with LSTa-BSA that has Neu5Acα2-3Gal linkage on sialyloligosaccharides on their surface. Those two strains could bind chicken RBCs. The PA titer of A/Osaka/18/2005 was 32 PA units for LSTa-BSA-latex. So these two viruses may recognize the Neu5Acα2-3Gal linkage rather than the NeuAcα2-6Gal sequence which is on the LSTc. Our results suggest that the latex particles LSTc-BSA CML can bind to influenza viruses and their binding is inhibited specifically by antigen specific antibody. The comparable sensitivity and specificity of PI to HI and its stability on storage are factors in favor of using LSTc-BSA CML to determine antigenicity of influenza viruses in a survey of epidemic strains. We also investigated whether influenza viruses isolated from avian feces bind NeuAcα2-6linkage on sialyloligosaccharides of LSTc-BSA-CML as well as NeuAcα2-3linkage which is known as a component of natural receptor. All five H10 subtype strains and a H7N7 strain showed similar agglutination pattern by LSTa-BSA-CML (which has only NeuAcα2-3Gal sequences) and

LSTc-BSA-CML (which has only NeuAcα2-6Gal sequences). Our data suggest that the receptor specificity of these avian influenza viruses did not differ. So the restriction of their ability to infect and replicate efficiently in mammalian cells may be placed on another point such as receptor affinity or extracellular inhibitors in the respiratory tract. These particles may be useful for investigation about receptor and viral ligand interactions.

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ORIGINAL ARTICLE

Influenza vaccine effectiveness in primary school children in Japan: a prospective cohort study using rapid diagnostic test results

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Abstract A low-cost, prospective cohort study using the results of rapid diagnostic test performed at local clinics was conducted to estimate influenza vaccine effectiveness (VE) in school children (6–12 year-olds). All children in four primary schools in Tsuchiura City, Ibaraki, Japan were enrolled ($n = 2607$). Vaccination status and other risk factors were obtained with a baseline questionnaire. Participants were encouraged to visit a clinic to have a rapid test when they developed an influenza-like illness during the winter season in 2006–2007, and 88.6% of those who reported influenza to the school had been tested. The result of the test was obtained with another questionnaire. The attack rate of influenza A and B was 5.4% and 11.9%, respectively. Logistic regression was used to model the association between influenza vaccination and rapid-test-confirmed influenza after adjusting for potential confounders. Influenza VE was calculated as (1− adjusted odds ratio) × 100. VE for total influenza was 21% (95% confidence interval −8 to 42), which was a combination of VE for influenza A (44%, 8–66) and VE for influenza B (5%, −37 to 34). Among several possibilities that would account for rather low VE estimates in this study, low sensitivity of the rapid test, and differential propensity to seek vaccination or medical care between the vaccinated and nonvaccinated were considered to be important. This study was able to estimate influenza VE at very low cost with high specificity in case ascertainment by collecting the readily available data on influenza rapid test with questionnaires.

Keywords Influenza · Vaccine effectiveness · Children · Rapid test

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Introduction

Influenza vaccination is the most effective method for preventing influenza virus infection and its potentially severe complications [1]. The vaccine effectiveness (VE) of trivalent inactivated vaccine to laboratory-confirmed influenza in healthy children has been reported as 59% [95% confidence interval (CI) 41–71) [2] or 67% (51–78) [3]. But influenza VE varies considerably by time, place, and the degree of antigenic distance between the vaccine strain and circulating strain. Therefore, it is important to take every opportunity to assess influenza VE to monitor the performance of a vaccination program.

In many studies assessing VE for influenza, influenza-like illness (ILI) has been used as the primary endpoint [2]. This is usually because virological confirmation of influenza infection is costly and logistically difficult. But ILI is not a specific case definition including other similar illnesses, and VE tend to be underestimated with such a low specific endpoint because of the diluting effect [4]. Influenza rapid diagnostic test (rapid test) has been used in routine medical practice in Japan since 1999. It has been reported to be more specific than clinical diagnosis during the influenza epidemic seasons [5]. However, there have been few reports on the use of rapid test for estimating influenza VE [6–8]. This would be because not all patients with ILI seek medical care, and not all those who attended a clinic are actually tested with a rapid test during influenza

seasons, which makes it almost impossible to assess influenza VE with a facility-based prospective cohort study. On the other hand, the Law of School Health in Japan prohibits a schoolchild who caught influenza from coming to school until 2 days after the febrile period, and this regulation gives a strong motivation to parents to take their febrile child to a clinic for a rapid test in winter. It is also of note that the cost of rapid test is mostly covered by public health insurance in Japan.

However, whereas most children with influenza are tested, those results have not been systematically collected for epidemiological purposes. This has been a waste of valuable information, and it could be corrected by involving school administrations into epidemiological practices for collection of these data. Therefore, we established a collaboration model in which several primary schools were invited to collect information on vaccination status and other risk factors of the children, as well as results of the rapid test from those who reported influenza, by using questionnaires. Here we report the results of estimated VE and the strength and limitations of the use of rapid test in assessing influenza VE in school children.

Methods

Study design and participants

This study was a prospective cohort study conducted during the 2006–2007 influenza season. To detect a significant effect of 50% VE when the incidence of influenza was assumed to be 6% and the vaccination coverage 50%, the required sample size was calculated as 1068 for each group (95% significance level with 90% power). Taking the average size of each school into account, the number of required schools was determined as four. Therefore, all pupils in four primary schools that were randomly selected from a total of 18 primary schools in Tsuchiura City (60 km northeast of Tokyo; population 143000), Ibaraki, Japan, were enrolled in the study ($n = 2607$).

Information collection and vaccination

A questionnaire for the baseline survey was sent to parents or guardians via school administrations in early January 2007, which inquired about potential confounders (age, sex, number of siblings, presence of underlying illness, and influenza disease history in the previous season) and vaccination status in the previous and current season. Influenza disease history in the current season up to the time of the baseline survey was also obtained. The vaccine contained A/New Caledonia/20/99(H1N1), A/Hiroshima/52/2005(H3N2), and B/Malaysia/2506/2004, and the antigen level for each strain was 30 μg/ml. Vaccine dosage was given according to the standard Japanese recommendation (i.e., 0.3 ml for schoolchildren aged 6–12 years). Two doses of vaccine were commonly administered subcutaneously 4 weeks apart between October and December 2006. Those who were vaccinated at least once during this period were considered as vaccinated in this study.

Case surveillance and definition

When a child was diagnosed as having influenza with a positive rapid test at a clinic, the child was forced to stay at home until 2 days after the febrile period according to the Law of School Health. When the child resumed school, the parent of the child was asked to submit an Influenza Reporting Form to the school administration for exemption purpose. At this time, another questionnaire was also given to the parent to collect information on the result of the rapid test. The primary endpoint of this study was a case of influenza confirmed by a positive rapid test (total influenza). A case was further classified as influenza A or B according to the test result, which was the secondary endpoint of this study. Those who were diagnosed as influenza without a rapid test (clinical influenza) were excluded from the main analysis, but they were included in a sensitivity analysis to assess the magnitude of their exclusion. This study was terminated at the end of the semester in March 2007.

Statistical analysis

Statistical analysis was performed with SAS 9.1 for Windows (SAS Institute, Cary, NC, USA). Univariate analysis was performed with a logistic regression model that included "vaccination of the current year" as an independent variable and "development of influenza" as a dependent variable. Adjusted VE was calculated as 1 minus odds ratio (OR) that was obtained from a logistic regression analysis accounting for potential confounders. Two models of logistic regression analysis were calculated. Model 1 was an unconditional logistic regression that included school, grade, sex, number of siblings, underlying illness, vaccination in the previous season, and corresponding type of influenza disease history in the previous season as potential confounders. Model 2 was a conditional logistic regression analysis that matched the individuals by school, grade and class while adjusting for the other confounders.

Ethical considerations

This study was approved by the board committee of the Tsuchiura City Medical Society and the Board of

Education of Tsuchiura City. Questionnaires were completed anonymously, and each child was identified by school ID number and birth date only.

Results

Participants

Of the 2607 schoolchildren in the four primary schools (designated as Schools A–D), response for the baseline survey was obtained from 2574 pupils (response rate 98.7%). Response rate did not vary significantly among the four schools (97.1–99.3%). Characteristics of those vaccinated and nonvaccinated are shown in Table 1. Age ranged from 6 to 12 years. Male to female ratio was 1.07 in total. A total of 1153 children (44.9%) reported to have had at least one dose of influenza vaccine in the 2006–2007 season. The age of the vaccinated children was significantly younger than that of the nonvaccinated children. Male to female ratio was significantly lower in vaccinated children. The number of siblings was lower in the vaccinated group. Children vaccinated in the current year were more likely to have had vaccinations in the previous year than nonvaccinated children (93.1% vs. 19.1%).

Vaccination and influenza incidence

The number of immunization doses and type-specific influenza cases in each school is shown in Table 2. No one reported suffering from influenza by the time of the baseline survey. The number of immunization doses varied significantly among four schools ($\chi^2 = 19.1$, $P = 0.004$). Fifty-five children (2.1%) reported to have been clinically diagnosed as influenza without the rapid test having been performed, which accounted for 11.4% [55/(55 + 429)] of all those who reported influenza. These 55 children were excluded from the main analyses. Six children reported to have been infected with both influenza A and B during the same season. There was a large epidemic of influenza B in school C (attack rate 18.6%). The overall attack rate of influenza A and B was 5.4% and 11.9%, respectively.

Weekly incidence of influenza

During the 2006–2007 winter season, there were epidemics of both influenza A and B in the study population (Fig. 1). The beginning of the influenza epidemics in this season was in late February. The high peak of influenza B between week 8 and 12 was largely accounted for by the epidemic in school C.

Vaccine effectiveness to each type of influenza

The OR of "vaccination in the current season" against development of each type of influenza was calculated with three different models (Table 3). All ORs were less than unity. Multivariate models resulted in smaller ORs than the univariate model, which suggested the presence of confounding effects toward null in some variables. When each potential confounder that was significantly associated with the exposure variable (i.e., grade, sex, number of siblings, and vaccination in the previous season) was removed from the unconditional logistic regression analysis one by one, only the removal of "vaccination in the previous season" gave a similar OR with that of the univariate analysis, which suggested that this variable was the most influential confounder (data not shown).

Table 1 Baseline characteristics of vaccinated and nonvaccinated children

	Vaccinated *n* (%) (*N* = 1153)	Nonvaccinated *n* (%) (*N* = 1413)	*P* value[a]
School grade			
1–2	450 (39.0)	419 (29.7)	<0.001
3–4	380 (33.0)	454 (32.1)	
5–6	323 (28.0)	540 (38.2)	
Male gender[b]	540 (46.8)	788 (55.8)	<0.001
No. of siblings: mean (SD)[c]	1.15 (0.73)	1.28 (0.89)	0.001
Underlying illness[d]	126 (11.1)	142 (10.3)	0.501
Vaccination in the previous season[e]	1074 (93.1)	270 (19.1)	<0.001
Influenza A in the previous season[f]	63 (5.5)	74 (5.2)	0.799
Influenza B in the previous season[f]	34 (2.9)	35 (2.5)	0.462

Data are expressed as *n* (%) unless otherwise indicated; eight children who did not provide information on immunization status are excluded

[a] χ^2 test, Wilcoxon rank sum test were used where appropriate

[b–f] 3, 8, 48, 9, and 45 children, respectively, who did not provide relevant information are excluded

Table 2 The number of immunization doses and incidence of influenza by school

	School				Total
	A	B	C	D	
No. of immunization dose					
0	340 (53.7)	357 (60.1)	382 (50.8)	334 (56.9)	1413 (55.1)
1	62 (9.8)	33 (5.6)	58 (7.7)	49 (8.3)	202 (7.9)
2	231 (36.5)	204 (34.3)	312 (41.5)	204 (34.8)	951 (37.1)
Outcome					
Rapid-test-confirmed influenza					
Total influenza[a]	86 (13.5)	78 (13.1)	178 (23.7)	87 (14.7)	429 (16.7)
Influenza A	40 (6.3)	26 (4.4)	33 (4.4)	30 (5.1)	129 (5.0)
Influenza B	46 (7.2)	51 (8.6)	140 (18.6)	57 (9.6)	294 (11.4)
Influenza A and B	0 (0.0)	1 (0.2)	5 (0.7)	0 (0.0)	6 (0.2)
Clinically diagnosed influenza	2 (0.3)	10 (1.7)	28 (3.7)	15 (2.5)	55 (2.1)
Not infected	548 (86.2)	507 (85.2)	546 (72.6)	489 (82.7)	2090 (81.2)
Total	636 (100)	595 (100)	752 (100)	591 (100)	2574 (100)

Eight children who did not provide information on immunization status are excluded. Figures in *parenthesis* are *column percents*

[a] Total influenza represents the sum of the following three rows

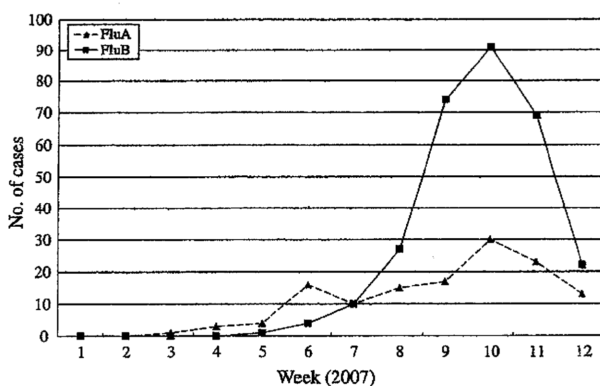

Fig. 1 Weekly incidence of influenza by type

Both model 1 (unconditional logistic regression without matching the class) and model 2 (conditional logistic regression with matching the class) gave similar results; hence, model 1 was used for the final result. The overall VE to total influenza was 21% (95% CI −8 to 42). The VE to influenza A and B was 44% (8–66) and 5% (−37 to 34), respectively.

Sensitivity analysis

To assess the magnitude of potential bias that may have resulted from excluding the 55 children who were clinically diagnosed as influenza without having a rapid test performed, unconditional logistic regression analysis was repeated with the 55 children included. The result of the OR for total influenza did not differ significantly (0.78, 95% CI 0.58–1.05).

Other risk factors for influenza

ORs of each variable for the primary (total influenza) and secondary endpoints (influenza A, B) derived from the unconditional logistic regression model are shown in Table 4. School grade and Number of siblings were converted to categorical variables in this analysis, whereas they were treated as continuous variables in the calculation for Table 3 (note that ORs of "vaccination in the current season" are slightly different between the two tables). None of the variables was significantly associated with the endpoint except for "vaccination in the current season" against influenza A (OR 0.57: 0.35–0.94). Marginally significant negative association was observed between "influenza A disease in the previous season" and "influenza A in the current season" (OR 0.27: 0.07–1.13). Positive association between "vaccination in the previous season" and influenza A was also marginally significant (OR 1.60: 0.98–2.60).

Discussion

In this study, we established a collaboration model that involved primary school administrations in order to collect necessary information for estimating influenza VE. Case ascertainment was based on the result of a rapid test conducted at local clinics, which was then obtained from the

Table 3 Odds ratio of vaccination against each type of influenza ($N = 2519$)

	Univariate		Multivariate			
			Unconditional logistic regression[a]		Conditional logistic regression[b]	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Total influenza	0.92 (0.75–1.13)	0.435	0.79 (0.58–1.08)	0.135	0.82 (0.60–1.12)	0.206
Influenza A	0.79 (0.55–1.12)	0.182	0.56 (0.34–0.92)	0.020	0.62 (0.38–1.03)	0.067
Influenza B	0.98 (0.77–1.25)	0.850	0.95 (0.66–1.37)	0.789	0.94 (0.65–1.36)	0.751

OR odds ratio, *CI* confidence interval

[a] Model includes school, grade (continuous variable), sex, number of siblings (continuous variable), underlying illness, vaccination in the previous season, corresponding type of influenza in the previous season, and vaccination in the current season

[b] Model includes sex, number of siblings (continuous variable), underlying illness, vaccination in the previous season, corresponding type of influenza in the previous season, and vaccination in the current season with matching by school, grade, and class

Table 4 Odds ratio (OR) and 95% confidence interval (CI) of each variable for the primary and secondary endpoint (unconditional logistic regression model: $N = 2519$)

	Total influenza		Influenza A		Influenza B	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Vaccination in the current season	0.80 (0.59–1.09)	0.154	0.57 (0.35–0.94)	0.027	0.96 (0.66–1.37)	0.804
School grade						
1–2	1.00		1.00		1.00	
3–4	0.85 (0.65–1.10)	0.206	0.97 (0.63–1.48)	0.882	0.83 (0.61–1.12)	0.227
5–6	0.86 (0.66–1.11)	0.249	0.85 (0.55–1.32)	0.470	0.89 (0.66–1.19)	0.422
	(Trend $P = 0.244$)		(Trend $P = 0.472$)		(Trend $P = 0.415$)	
Male	1.06 (0.85–1.31)	0.606	0.84 (0.59–1.20)	0.337	1.21 (0.94–1.55)	0.141
Number of siblings						
No. sibling	1.00		1.00		1.00	
1	0.86 (0.64–1.17)	0.343	0.66 (0.40–1.08)	0.097	0.97 (0.68–1.38)	0.844
2+	1.01 (0.73–1.40)	0.958	1.15 (0.70–1.91)	0.581	0.91 (0.62–1.34)	0.630
	(Trend $P = 0.691$)		(Trend $P = 0.204$)		(Trend $P = 0.605$)	
Underlying illness	0.98 (0.69–1.38)	0.896	0.60 (0.30–1.20)	0.149	1.20 (0.82–1.75)	0.357
Vaccination in the previous season	1.16 (0.86–1.59)	0.333	1.60 (0.98–2.60)	0.059	0.96 (0.67–1.38)	0.824
Corresponding type of influenza in the previous season	0.87 (0.57–1.33)	0.517	0.27 (0.07–1.13)	0.072	1.20 (0.58–2.47)	0.628

Model includes school and all variables in tables

parent with a questionnaire. This study design has several advantages. First, all pupils, both vaccinated and nonvaccinated, were equally followed up for the development of ILI (including influenza) by the school administration. In a prospective cohort study, this equal intensity in follow-up between exposed and unexposed individuals is imperative. Second, follow-up was almost perfect because children come to school anyway. If there is a large number of losses to follow-up, the power of the study would be weakened, or the result could be even biased if the loss was differential. Third, this study used the result of rapid tests as the endpoint, which is far more specific than the diagnosis of ILI [5]. This high specificity in case ascertainment is important in reducing the misclassification of outcome, which would lead to a lower estimate of VE by diluting effect [4]. Fourth, the cost of this study was minimal because we collected results of the rapid test that were readily available at home, and the means of data collection was a simple questionnaire that was distributed to parents through the school administration. This low cost also contributed to making the sample size large enough.

On the other hand, there are several disadvantages inherent in this study design. First, a major premise was that every participant who developed ILI during the

influenza epidemic season went to a clinic and was checked with a rapid test. Usually, this condition is not met [7], because not all febrile patients seek medical care, and even if they visited a clinic, not all of them would be checked with a rapid test even during influenza seasons. However, primary school administrations in Japan are so concerned with influenza in the fear of the potential risk of severe complications that they tend to give a strong recommendation to parents to take a febrile child to a clinic during influenza seasons, where rapid test is commonly available without much cost. School exemption policy that allows pupils with influenza to absent themselves is another motivation for parents to have the child tested and report the result to the school. In fact, 88.6% [429/(429 + 55)] of children who visited a clinic were checked with a rapid test in this study (Table 2). Therefore, we believe that compliance of the study participants was high enough to produce a reliable estimate of VE. However, this study design would not be feasible in parts of the world in which rapid test is not a routine medical practice.

In a review of randomized clinical studies assessing influenza VE in healthy children (≤18 years), Manzoli et al. [3] reported 67% (51–78%) of VE for laboratory-confirmed influenza. Similarly, Smith et al. [2] reported 59% (41–71%) of VE among healthy children (≥2 years) in their meta-analysis. In our study, the point estimate of adjusted VE for total influenza was 21%, and that for influenza A and B was 44% and 5%, respectively (Table 3). Rather low estimates of our results may have arisen from some other disadvantages of this study design as followings.

1. Sensitivity of the influenza rapid test is known to be lower than its specificity [5–9]. It is possible that some children who developed influenza were misclassified as noninfluenza because of low test sensitivity, and such misclassification would lead to a lower estimate of VE by the diluting effect [4].
2. If a child with mild influenza did not go to a clinic and recovered at home, he or she would be misclassified as not infected. If such a child were more common in the nonvaccinated group, the estimate of VE would be biased toward null. In fact, working parents are often too busy to take a child to a vaccination clinic, and tend not to be able to take a febrile child to a clinic for a rapid test. This differential propensity to seek medical care or vaccination between the vaccinated and nonvaccinated could have biased our results, but we were not able to assess this possibility because at the end of the study period, we did not administer, for logistical reasons, another questionnaire targeting all the children to confirm whether they had experienced febrile episodes and whether they actually visited a clinic for a rapid test. Those who did not submit an Influenza Reporting Form to the school were automatically considered not infected, and this was a major limitation.
3. Misclassification of exposure status (vaccination) can also bias the estimate of VE. However, its effect in this study should not be remarkable, because the baseline survey was conducted in early January 2007 when the usual vaccination period for influenza had just ended. Therefore, parents' recall bias on the vaccination status of their children would not have been so significant.
4. To assess influenza VE in a cohort study, it is important that both vaccinated and nonvaccinated groups are equally exposed to influenza virus during the study period, because virus exposure is necessary to develop influenza. However, some classes in a school experience influenza outbreaks, whereas others do not. To adjust the degree of viral exposure by the class, we also performed a conditional logistic regression analysis that matched the class, grade, and school (Table 3). However, the result was almost identical with that of the unconditional logistic regression analysis. Therefore, differential viral exposure by the class did not seem to play a major role in affecting VE.
5. Orenstein et al. [10] reported that the major determinants of bias in estimating the influenza VE with the rapid test results were test specificity and ratio of influenza attack rate compared with noninfluenza ILI. If the number of noninfluenza ILI was large during the season, many children would be falsely diagnosed as positive with a rapid test of imperfect specificity, and these false positives would lower the VE estimates by the diluting effect. However, as we did not obtain information on noninfluenza ILI, we were unable to assess this possibility.
6. We did not check the validity of our results by conducting a subsidiary study in which small validation sets that use a more specific outcome measurement, such as virus isolation, are recruited [11]. Although VE for influenza B was previously reported as rather low in Japan [12], we were not certain whether our estimate of VE for influenza B (5%, −37 to 34) was reliable. This wide confidence interval may suggest the lack of study power, and the true value might have been higher than 5%, because the National Institute of Health, Japan, reported that the circulating strain of influenza B was antigenically well matched with the vaccine strain in 2006–2007 season [13].

Table 4 shows OR of each variable against each endpoint. Although not statistically significant, "vaccination in the previous season" was positively associated with "influenza A in the current season" (OR 1.60: 0.98–2.60),

and "influenza A in the previous season" was negatively associated with "influenza A in the current season" (OR 0.27: 0.07–1.13), respectively. It is rather difficult to interpret the former association, although several investigators have discussed the efficacy of annual revaccination against influenza [14, 15]. There may be an unknown confounder that enhances development of influenza A and is also associated with the vaccination behavior of the parents, although this study failed to show such evidence. The latter association might suggest that natural immunity elicited by infection with influenza A in 2005–2006 continued to protect the host in 2006–2007.

In conclusion, the use of rapid test can be a valuable tool in estimating type-specific influenza VE in a large cohort if the test is a routine practice and all patients with ILI go to a clinic for the test. Primary schools in Japan meet these requirements and provide a good opportunity to conduct a VE study at a low cost. Lack of the validity check in this study design would recommend a subsidiary study to compare the estimated VE with the one from a validation set within the same population that uses laboratory-confirmed influenza as an endpoint. We also recommend repeating the same study in various areas in Japan for consecutive years to confirm the feasibility and validity of this study design and monitor the efficacy of the vaccination program over time.

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Development of an Immunochromatographic Assay Specifically Detecting Pandemic H1N1 (2009) Influenza Virus[▽]

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The pandemic caused by a new type of influenza virus, pandemic H1N1 (2009) influenza virus A (AH1pdm), has had a major worldwide impact. Since hemagglutinin (HA) genes are among the most specific genes in the influenza virus genome, AH1pdm can be definitively diagnosed by viral gene analysis targeting the HA genes. This type of analysis, however, cannot be easily performed in clinical settings. While commercially available rapid diagnosis kits (RDKs) based on immunochromatography can be used to detect nucleoproteins (NPs) of influenza A and B viruses in clinical samples, there are no such kits that are specific for AH1pdm. We show here that an RDK using a combination of monoclonal antibodies against NP can be used to specifically detect AH1pdm. The RDK recognized AH1pdm virus isolates but did not recognize seasonal H1N1 and H3N2 and influenza B viruses, indicating that the specificity of the RDK is 100%. A parallel comparison of RDK with a commercial influenza A/B virus kit revealed that both types of kits had equal sensitivities in detecting their respective viruses. Preliminary evaluation of clinical samples from 5 individuals with PCR-confirmed human AH1pdm infection showed that the RDK was positive for all samples, with the same detection intensity as that of a commercial influenza A/B virus kit. This RDK, together with a new vaccine and the stockpiling of anti-influenza drugs, will make aggressive measures to contain AH1pdm infections possible.

The pandemic caused by a new type of influenza virus, pandemic H1N1 (2009) influenza virus A (AH1pdm), has had a major worldwide impact. As of 27 September 2009, more than 4,100 deaths from AH1pdm infection have been reported to the World Health Organization (WHO) (http://www.who.int/csr/don/2009_10_02/en/index.html). Current methods used to diagnose AH1pdm virus in clinical specimens are based on viral RNA analysis targeting hemagglutinin (HA) genes, because the HA genes are among the most specific genes in the influenza virus genome. Although these methods are highly sensitive, they usually take more than 2 to 6 h to complete and require well-equipped laboratories with virologists or well-trained medical technicians and specialized tools for virus genome isolation and amplification (6, 8) (http://www.who.int/entity/csr/resources/publications/swineflu/CDCRealtimeRTPCR_SwineH1Assay-2009_20090430.pdf). Rapid diagnostic kits (RDKs) based on immunochromatography utilize antibodies (Abs) against antigens of interest. Although RDKs are usually less sensitive than genetic assays, they do not require the isolation of a viral genome, thus overcoming the intrinsic difficulties of viral gene analyses. RDKs for many infectious diseases (2, 4, 9, 11–14), including influenza viruses A and B (1), are commercially available. However, RDKs capable of distinguishing AH1pdm viruses from seasonal influenza viruses have yet to be implemented in a clinical setting.

Nucleoproteins (NPs) of influenza A, B, and C viruses have important differences in their antigenicities that enable them to be distinguished from one another but are highly conserved within each major serotype. Thus, antibodies to NPs have been utilized in commercially available RDKs to distinguish between influenza A and B viruses (15). In a monoclonal antibody (MAb) preparation procedure targeting NPs derived from highly pathogenic H5N1 avian influenza (HPAI), we obtained 2 MAbs that reacted with NPs of AH1pdm as well as that of HPAI but not those of seasonal influenza A virus. We have therefore utilized these MAbs in the development of novel RDKs for AH1pdm, and we have validated these RDKs in laboratory environments.

MATERIALS AND METHODS

Monoclonal antibodies to influenza A virus nucleoprotein (NP). Recombinant NP of influenza A virus [A/Viet Nam/VL-020/2005 (H5N1)] (GenBank accession number AAZ72762), a virus isolated from a patient infected with HPAI, was prepared from *Escherichia coli* BL21(DE3) CodonPlus-RIPL cells (Stratagene), which carry a TAGZyme pQE2 (Qiagen) derivative carrying the NP protein gene (7). The NP was used to immunize 7- to 9-week-old female WKY rats (Oriental Yeast Co., Ltd.), and rat MAbs were prepared as described previously (10).

ELISA analysis of MAbs. The reactivity of the MAbs with NPs derived from seasonal influenza and AH1pdm was analyzed by conventional enzyme-linked immunosorbent assay (ELISA) using microplates coated with NPs or by sandwich ELISA using microplates coated with polyclonal antibodies prepared from rabbits immunized with recombinant NPs.

Sources of NPs for the sandwich ELISA included cultured human A/New York/55/2004 (H3N2) and A/New Caledonia/20/1999 (H1N1) viruses in tissue culture and recombinant NPs from HEK293 cells transfected with cytomegalovirus (CMV) promoter-driven plasmids (7) carrying an NP gene with the sequence of A/California/04/2009 (H1N1) (GenBank accession number ACP44151), a virus isolated from a patient infected with AH1pdm; that of H5N1 HPAI virus [A/Viet Nam/VL-020/2005 (H5N1)] (accession number AAZ72762), a virus isolated from a patient infected with HPAI; and chimeric NPs derived from those of H5N1 HPAI and seasonal H3N2 viruses (as described above) (see

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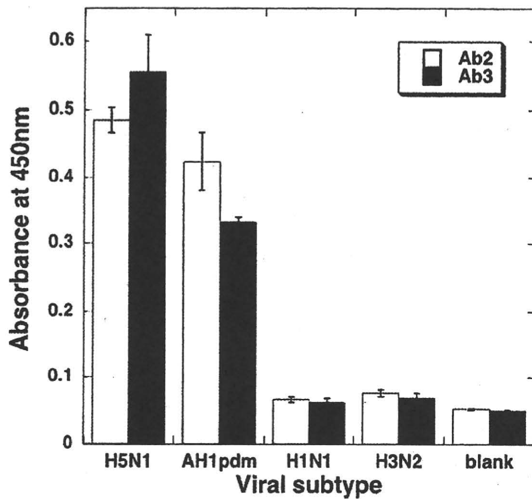

FIG. 1. Reactivity of Ab2 and Ab3 against NPs from AH1pdm, H5N1 HPAI, seasonal H1N1, and seasonal H3N2 viruses in a sandwich ELISA. After fixing the capture polyclonal antibody, roughly 10 ng of each NP was captured on the plates, and about 50 ng of each MAb was added to the wells. Data are presented as means ± standard errors (SE).

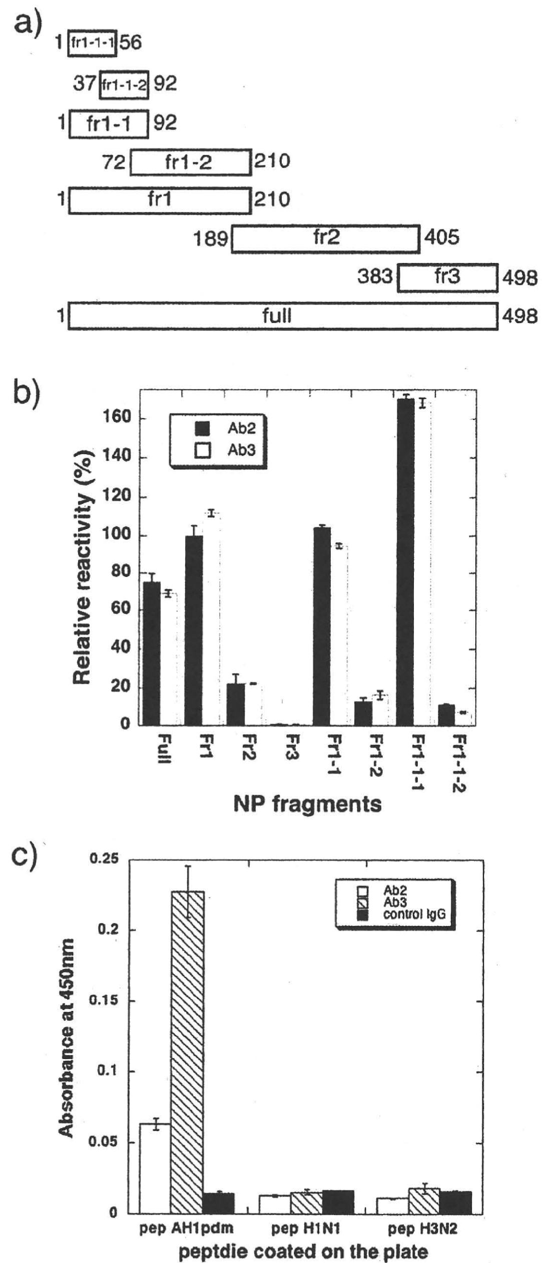

FIG. 2. Epitope mapping of Ab2 and Ab3 by conventional ELISA. (a) Recombinant fragments derived from the NP of AH1pdm virus used to map Ab2 and Ab3 epitopes. (b) Reactivity of fragments derived from NP with Ab2 and Ab3 as determined by conventional ELISA. Fifty nanograms of each fragment was added to the wells, and the plates were reacted with 50 ng/well of MAb. Data were normalized against the positive control (polyclonal rat anti-NP Ab). (c) Reactivity of synthetic peptides with Ab2 and Ab3, with rat IgG as the negative control, in a conventional ELISA. Five hundreds nanograms of each peptide was captured on the wells, and 500 ng/well of MAb was added. Data in b and c are presented as means ± SE.

Fig. 3b). To construct chimeric NPs, three regions of each NP were amplified separately by PCR using the primers containing restriction sites and *ExTaq* polymerase (Takara). Primer sequences are available upon request. The PCR products were subsequently purified, digested with the corresponding restriction enzymes, and ligated to insert the DNA fragments into the expression plasmid as described above.

The concentration of each NP was normalized by conventional Western blotting with rabbit anti-NP polyclonal antibody. To perform sandwich ELISA, 250 ng of rabbit anti-NP polyclonal Ab dissolved in 50 mM sodium-carbonate buffer (pH 9.0) was fixed to each well of a 96-well microtiter plate (Corning) at room temperature for 1 h. After washing with phosphate-buffered saline containing 0.02% Tween 20 (PBS-T) and blocking with SuperBlock (Pierce), 10 ng of the NPs dissolved in PBS-T was added to each well. Following incubation and washing, the wells were incubated with culture supernatants of hybridomas producing the indicated MAbs. In a conventional ELISA, 50 ng/well of antigens was fixed onto the plates directly. The binding of MAbs was detected with horseradish peroxidase (HRP)-goat anti-rat IgG (GE Healthcare) and tetramethylbenzidine (TMB) (Bio-Rad).

Epitope mapping of MAb using recombinant NP fragments. Eight NP fragments (see Fig. 2a) derived from NP [A/California/04/2009 (H1N1)] (GenBank accession number ACP44151) were prepared in *E. coli* cells as described above and used for the epitope mapping of MAbs based on ELISA results. Synthetic peptides prepared by a commercial service (>70% purity; Invitrogen) (500 ng each) were fixed onto the plates by incubation in 50 mM carbonate buffer (pH 9.0) containing the chemical cross-linker disuccinimidyl suberate (DSS) (1 mM; Pierce) at room temperature for 1 h, followed by epitope mapping.

Assembly of RDK for AH1pdm. The RDK was assembled based on the Quick-chaser Flu A,B test (Mizuho Medy, Japan), a commercially available rapid diagnosis kit used to detect influenza A and B viruses in clinical specimens. Sample migration was assayed by using rabbit immunoglobulin (rIg) (Rockland) and anti-rabbit IgG, with rIg conjugated to gold particles as the mobile phase (3). Ab1 recognizes NPs from AH1pdm, seasonal H1N1, and H3N2 viruses but not NP from HPAI virus, while Ab2 and Ab3 recognize NPs from AH1pdm and HPAI viruses but not NPs from seasonal H1N1 or H3N2 virus (see Results). To detect AH1pdm specifically and to exclude the reactivity against NP from HPAI virus in the RDK, Ab1 was used as the gold particle-labeled mobile antibody, while Ab2 or Ab3 was used as the capture antibody. In such a composition of the RDK, NP from H5N1 HPAI virus will migrate to and bind to Ab2 or Ab3, although it will not generate a line because it is not attached to colloidal gold-labeled Ab1. To prepare test lines, Ab2 or Ab3 (0.76 μg/test) was coated onto nitrocellulose membranes (Millipore) at a position 30 mm from the sample dropping point and allowed to dry at room temperature. To prepare control lines, anti-rabbit IgG (0.2 μg/test) (Rockland) was coated onto the membrane at a position 39 mm from the sample dropping point and allowed to dry. Pads were prepared by dropping anti-influenza A virus NP MAb (clone M322211; Fitzgerald) (named Ab1 in this study) and rIg, each conjugated with colloidal gold, onto glass filters, followed by freeze-drying. The nitrocellulose membrane and pad were assembled with filter papers as sample application pads and absorption

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TABLE 1. Prevalence of sequence type at amino acids 16 to 18 in NPs from AH1pdm, HPAI (H5N1), seasonal H1N1, and seasonal H3N2 influenza viruses isolated from infected patients[a]

Virus	GGE		DGE		DGD		XGE		SGE		Total no. of isolates
	No. of isolates	% of isolates	No. of isolates	% of isolates	No. of isolates	% of isolates	No. of isolates	% of isolates	No. of isolates	% of isolates	
Seasonal H3N2	1	0.2	0	0.0	251	100.0	0	0.0	1	33.3	253
Seasonal H1N1	0	0.0	373	99.2	0	0.0	0	0.0	1	33.3	374
HPAI H5N1	14	2.5	0	0.0	0	0.0	0	0.0	0	0.0	14
AH1pdm	537	97.3	3	0.8	0	0.0	2	100.0	1	33.3	543
Total	552	100.0	376	100.0	251	100.0	2	100.0	3	100.0	1,184

[a] The prevalence of each type of influenza A virus was summarized based on data accumulated for the Influenza Virus Resource at NCBI (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) during 1 January 2007 to 11 September 2009.

pads on a plate within a plastic housing (see Fig. 4). These assembled RDKs were stored in a waterproof bag with desiccant at room temperature (1°C to 30°C).

Assessment of RDK. The specificity of the RDK for AH1pdm was assessed by using reverse transcription (RT)-PCR-confirmed AH1pdm and seasonal influenza A and B viral strains isolated during 2009 from infected individuals in Japan by culturing on Madin-Darby canine kidney (MDCK) cells. The viral isolation procedures were performed at the Osaka Prefectural Institute of Public Health. Experiments using clinical samples were reviewed and approved by the Institutional Review Board of Mizuho Medy. All patients provided written informed consent, and all clinical samples were assayed anonymously.

Each isolate (ca. 10^8 viral copies/100 μl) was extracted into sample extraction tubes containing sample extraction buffer (0.4 M Tris buffer containing 1% nonionic detergent and 0.09% sodium azide) and assayed by RDK by visual assessment of line intensity on a scale from − to +++. In parallel, these samples were analyzed with a commercial influenza A/B virus kit to validate reactivity. In addition, these samples were diluted (see Table 2) to assess the sensitivity of the RDK for AH1pdm. The specificity of the RDKs was also tested by using clinical specimens obtained by nasal swabs.

Analyses of viral copy numbers and detection of the influenza viruses by real-time RT-PCR were performed according to WHO criteria (http://www.who.int/csr/resources/publications/swineflu/WHO_Diagnostic_RecommendationsH1N1_20090521.pdf) for AH1pdm and influenza A viruses and according to methods described previously by van Elden et al. (16) for influenza B virus.

RESULTS

Monoclonal antibodies recognizing NP of AH1pdm. During the preparation of MAbs against NP of HPAI virus, we obtained 2 MAbs, Ab2 and Ab3, that were highly reactive against NP from AH1pdm as well as HPAI virus but not against NPs from seasonal influenza A H1N1 [A/New Caledonia/20/1999 (H1N1)] and H3N2 [A/New York/55/2004 (H3N2)] viruses (Fig. 1a). Both MAbs showed 6-fold-greater reactivity with NP from AH1pdm than with NPs from the seasonal H1N1 and H3N2 viruses.

To identify the determinant(s) recognized by Ab2 and Ab3 in the AH1pdm virus NP, we performed epitope mapping with 8 protein fragments derived from the NP of AH1pdm virus (Fig. 2). Both Ab2 and Ab3 reacted with the most N-terminal fragment, fr 1-1-1, containing amino acids (aa) 1 to 56 of NP (Fig. 2b) only. Since amino acids 37 to 56 are overlapped with those of fr 1-1-2, we compared the amino acid sequences of amino acids 1 to 36 of NPs from seasonal H3N2, seasonal H1N1 HPAI, and AH1pdm viruses. As a result, we found mutations specific for HPAI and AH1pdm viruses at amino acid positions 16 to 18. NPs from AH1pdm and H5N1 HPAI viruses have the sequence GGE, whereas NPs from the seasonal H1N1 and H3N2 viruses have the sequences DGE and DGD, respectively. To confirm that Ab2 and Ab3 could distinguish the GGE sequence from DGE and DGD, we synthesized three 15-mer peptides, QGTKRSYEQMETDGE (peptide H1 from seasonal H1N1 virus), QGTKRSYEQMETDGD (peptide H3 from seasonal H3N2 virus), and QGTKRSYEQMETGGE (peptide AH1pdm from AH1pdm and HPAI viruses) and analyzed their reactivities with Ab2 and Ab3 (Fig. 2c). Both Ab2 and Ab3 reacted with peptide AH1pdm but not with seasonal peptides H1 and H3.

To analyze the prevalence of the each sequence in NPs of influenza A virus from human cases, we analyzed the amino acid sequences of NPs from more than 1,000 patients infected from 2007 to 2009, including patients infected with seasonal H3N2, seasonal H1N1, HPAI, and AH1pdm viruses (Table 1). Of the 543 AH1pdm virus isolates, 537 (97.3%) had the sequence GGE, whereas only 6 AH1pdm virus isolates had sequences other than GGE. All 14 HPAI H5N1 virus isolates from human cases also had the sequence GGE. In comparison, only one seasonal influenza isolate (a seasonal H3N2 virus) had the sequence GGE. These results indicate that Ab2 and Ab3 can be used to distinguish AH1pdm in addition to HPAI viruses from seasonal H1N1 and seasonal H3N2 viruses by recognizing amino acids 16 to 18, located at the N termini of NPs.

Monoclonal antibodies not recognizing NP of H5N1 HPAI virus. We also unexpectedly found a commercial MAb, named Ab1, that failed to recognize NP of H5N1 HPAI virus but that was capable of recognizing NPs of AH1pdm and seasonal H1N1 and H3N2 viruses (Fig. 3a). Ab1 showed very little reactivity with NP from H5N1 virus, the same as blank. Although we attempted to identify the epitope as we did for Ab2 and Ab3 by using a fragmented NP as shown in Fig. 2b, Ab1 did not react with the fragments at all (data not shown). Thus, we prepared chimeric NPs of H5N1 and H3N2 viruses (Fig. 3b) as the recombinant proteins in an expression system using HEK293 cells, and their reactivity with Ab1 was analyzed. Ab1 reacted with chimera 2 and chimera 4 as well as NP from H3N2 virus but failed to react with chimera 1 and chimera 3. These results indicated that Ab1 is a MAb recognizing a conformational epitope of NPs that appears to be located between amino acids 1 and 188. This conclusion is based on the fact that Ab1 recognized chimeras containing aa 1 to 188 from viruses that reacted with Ab1 (e.g., H3N2 virus), while Ab1 failed to recognize chimeras that contained aa 1 to 188 from viruses that failed to react with Ab1 (e.g., H5N1 virus).

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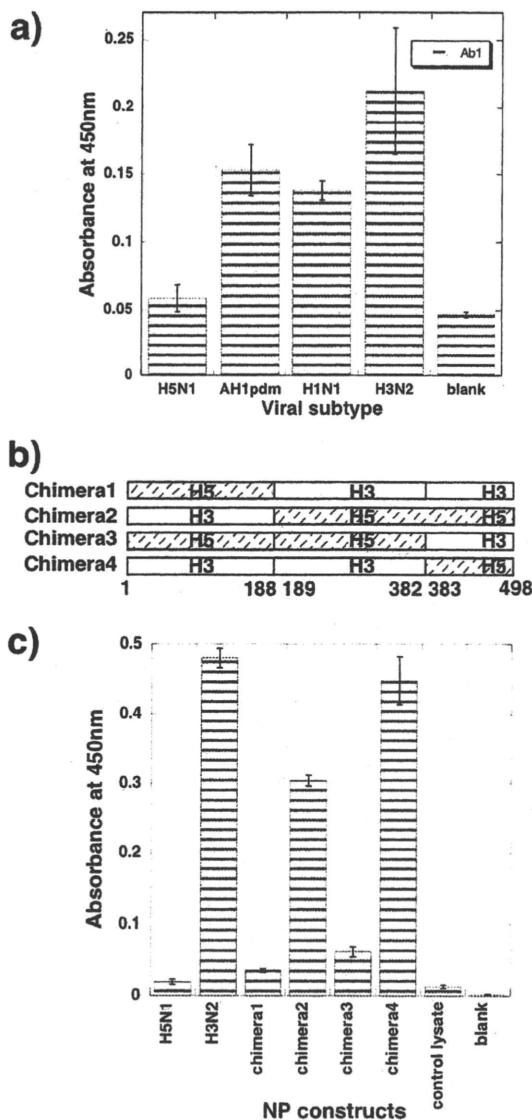

FIG. 3. Reactivity of Ab1 against NPs from AH1pdm, H5N1 HPAI, seasonal H1N1, and seasonal H3N2 viruses in a sandwich ELISA (a) and epitope analysis of Ab1 using chimeric NP (b and c). (a) After fixing the capture polyclonal antibody, roughly 10 ng of each NP was captured on the plates, and about 50 ng of MAb was added to the wells. Data are presented as means ± SE. (b) Structure of chimeric NPs used in this study derived from NPs of H5N1 HPAI and seasonal H3N2 viruses. (c) Reactivity of chimeric NPs with Ab1. After fixing the capture polyclonal antibody, roughly 10 ng of each chimera was captured on the plates, and 50 ng of Ab1 was added to the wells. Data are presented as means ± SE.

Development of RDK and evaluation using isolated influenza viruses. As shown above, Ab2 and Ab3 reacted with NP from AH1pdm and HPAI virus, while Ab1 reacted with NP from AH1pdm and seasonal H1N1 and H3N2 viruses but failed to react with NP from HPAI virus. These observations suggested that a combination of Ab1, Ab2, and Ab3 could be used to distinguish NPs derived from AH1pdm viruses from those of seasonal influenza and H5N1 HPAI viruses. We tested the feasibility of utilizing prototype RDKs.

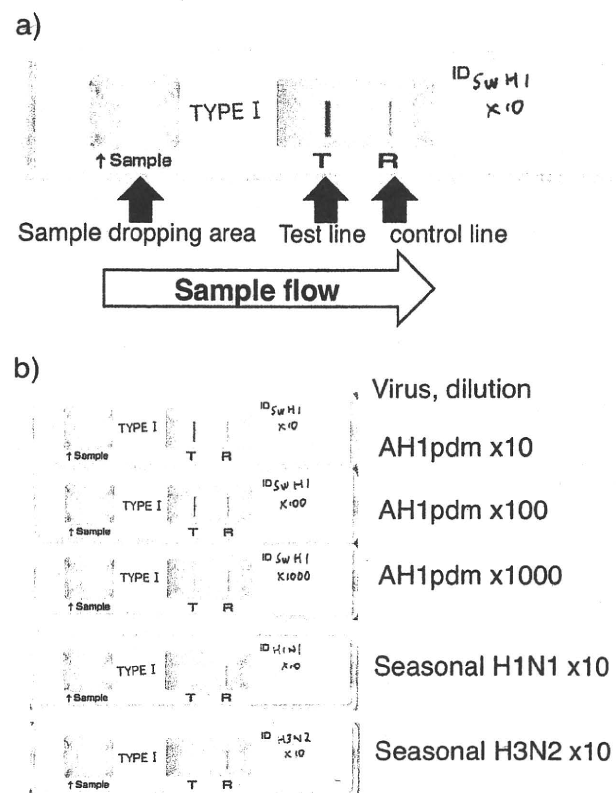

FIG. 4. Prototype RDK and specific detection of AH1pdm virus. (a) Composition of the RDK. Each RDK consists of a sample dropping area and a detection area containing a test line and a control line. The direction of sample flow is indicated. (b) Representative results of RDK dropped samples. One hundred microliters of diluted AH1pdm or seasonal influenza A virus samples (ca. 1×10^8 viral copies/100 μl) was dropped onto RDKs.

Prototype RDKs for the immunochromatographic detection of AH1pdm virus using MAbs were assembled as described in Materials and Methods, based on a commercial influenza A/B virus kit (Fig. 4a). These RDKs were used to detect viruses by dropping 100 μl of each diluted sample (in extraction buffer) onto the test plate and waiting for 10 min (Fig. 4b). A positive result for the AH1pdm virus was indicated by a purple line in the test line area as well as by the control line, which was included to ensure a normal flow of reaction mixture. The intensity of the test lines was scored from − to +++, whereas the control lines were scored ++ (Fig. 4b), making the assay semiquantitative. The RDKs showed positive results with the AH1pdm virus sample, even after a 1,000-fold dilution, but not with seasonal influenza virus samples even at 10-fold dilutions. We also tested the reactivity of our RDKs with A/New Jersey/8/76, obtained from the ATCC (ATCC VR-897), which is known as the prototype of swine influenza virus transmitted to humans. The viral stain harbors the GGE sequence in its NP and reacted with the prototype kits, as expected (data not shown).

Sensitivity of the prototype kits for AH1pdm virus. To assess the sensitivity of the prototype kits prepared using Ab2 or Ab3, cultured influenza A virus samples were diluted and dropped onto the kits; in parallel, these samples were tested by using a

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TABLE 2. Sensitivity of prototype RDKs using Ab2 or Ab3 against a cultured AH1pdm virus strain[a]

Virus	Dilution rate	Sensitivity		
		RDK1 (Ab2)	RDK2 (Ab3)	Commercial kit for influenza A/B virus
AH1pdm	×100	+++	+++	+++
	×500	+++	+++	+++
	×1,000	+++	+++	+++
	×2,000	+++	+++	+++
	×4,000	++	++	++
	×8,000	++	++	++
	×16,000	+	+	+
	×32,000	−	−	−
	×64,000	−	−	−
Seasonal H1N1	×10	−	−	+++
Seasonal H3N2	×10	−	−	+++

[a] Cultured AH1pdm virus, seasonal H1N1 virus, or seasonal H3N2 virus (ca. 10^9 viral copies/100μl) was diluted as indicated and dropped onto the prototype RDKs or commercial influenza A/B virus kits for influenza A/B virus. The detection limit of the commercial kit (Quickchaser Flu A/B; Mizuho Medy Co., Ltd) for influenza A/B virus was less than 2.2×10^4 50% tissue culture infective doses ($TCID_{50}s$)/kit.

commercial influenza A/B virus kit, which cannot distinguish AH1pdm from other seasonal influenza A viruses (Table 2). The prototype kits reacted positively with AH1pdm samples diluted 1:16,000, similar to the sensitivity of the commercial kits for seasonal influenza A viruses. In contrast, the prototype kits did not show positive reactions with any of the seasonal influenza A virus samples, even at a 1:10 dilution, while the commercial kits did. The RDKs had an average lower detection threshold for AH1pdm of 2×10^5 viral copies/kit based on analyses using five different AH1pdm viral cultures (data not shown).

Specificity of the prototype kits for AH1pdm virus. To evaluate the specificity of our prototype RDK in detecting AH1pdm viruses, influenza viruses isolated in tissue culture during 2009 were screened. Specifically, we assessed 30 AH1pdm, 20 seasonal H1N1, 20 seasonal H3N2, and 5 influenza B viruses independently isolated from infected patients. The RDK was positive for all the AH1pdm virus isolates (Table 3), with the intensity of the lines essentially identical to those observed with commercial influenza A/B virus kits (data not shown). In contrast, the RDK did not react with any of the H1N1, H3N2, and influenza B virus isolates. These results indicate that our prototype RDK could distinguish AH1pdm from seasonal influenza viruses with a specificity of 100%.

TABLE 3. Specificity of prototype RDKs prepared with Ab2 or Ab3 using cultured influenza virus isolates[a]

Virus type	No. of positive samples/total no. of samples		
	Kit 1 (Ab2)	Kit 2 (Ab3)	Commercial kit for influenza A/B virus
AH1pdm	30/30	30/30	30/30
H1N1	0/20	0/20	20/20
H3N2	0/20	0/20	20/20
Influenza B virus	0/5	0/5	5/5

[a] Influenza viruses (30 AH1pdm, 20 H1N1, 20 H3N2, and 5 influenza B virus isolates) isolated from infected patients during 2009 by tissue culture were tested for their reactivities with prototype kits and a commercial influenza A/B virus kit.

TABLE 4. Specificity of prototype RDKs prepared with Ab2 or Ab3 using clinical specimens[a]

Virus type	No. of positive samples/total no. of samples		
	Kit 1 (Ab2)	Kit 2 (Ab3)	Commercial kit for influenza A/B virus
AH1pdm	5/5[b]	5/5[b]	5/5[b]
Seasonal influenza A virus	0/20	0/20	20/20
Seasonal influenza B virus	0/9	0/9	9/9
Influenza virus negative	0/20	0/20	0/20

[a] Clinical specimens (all obtained by nasal swabs) positive for AH1pdm (n = 5), seasonal influenza A virus (n = 20), and seasonal influenza B virus (n = 9) or negative for influenza virus by PCR analysis were applied to prototype kits and commercial influenza A/B virus kits.

[b] The intensities for each type of sample were essentially the same.

We also tested the reactivities of the RDKs using clinical specimens obtained during 2009 in Japan from 5 AH1pdm, 20 seasonal influenza A, and 9 seasonal influenza B virus-infected individuals as well as with 20 clinical specimens that were negative for influenza virus based on PCR analyses (Table 4). The RDKs reacted with all 5 AH1pdm virus-positive samples but not with any of the seasonal influenza A virus-, seasonal influenza B virus-, and influenza virus-negative samples. In comparison, the commercial influenza A/B virus kits showed positive reactions with all of the AH1pdm, seasonal influenza A, and seasonal influenza B virus clinical samples. These results indicate that our RDKs could specifically detect AH1pdm in clinical samples.

DISCUSSION

Here we have described the development of an RDK that can be used to distinguish AH1pdm viruses from seasonal influenza viruses using MAbs Ab2 and Ab3. NPs of influenza A, B, and C viruses have important differences in their antigenicities that enable them to be distinguished from one another but are highly conserved within each major serotype. A detailed analysis of the NPs from influenza A virus, however, showed considerable sequence variation among them (data not shown). Epitope mapping of Ab2 and Ab3 showed that both MAbs recognize a peptide containing residues 16 to 18 of NP from AH1pdm as well as H5N1 HPAI viruses. The corresponding region of NPs from AH1pdm and H5N1 HPAI viruses was GGE, while those of seasonal H1N1 and H3N2 viruses were DGE and DGD, respectively. Prevalence analyses indicated that this amino acid difference could be used to distinguish AH1pdm from seasonal influenza A viruses (Table 1). Usually, the detection of new-type influenza viruses, such as AH1pdm and H5N1 viruses, in clinical specimens is performed with assays targeting HA of influenza virus (http://www.who.int/entity/csr/resources/publications/swineflu/CDCRealtimeRTPCR_SwineH1Assay-2009_20090430.pdf) because of the subtype specificity of HA genes. Since HA is highly mutagenic (5), assays of this gene would have to take into account mutations in HA that occur every season. In contrast, the

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amino acid sequences of the NPs, including that of the AH1pdm virus, which emerged in 2009, are, in general, well conserved. Although reassorted viruses may emerge, from which NPs of specific viruses of swine origin may be lost, our results indicate that methods targeting influenza virus NPs to discriminate among viral subtypes are plausible alternatives to HA-directed methods.

The RDK developed in this study represents a means for the rapid, noninvasive, and cost-effective diagnosis of AH1pdm virus in infected individuals by health workers in remote sites, at the bedside, and in quarantined areas such as airports. The use of the RDK not only could reduce the risk of mortality and morbidity but also would reduce the impact of an influenza pandemic by facilitating the more rapid diagnosis, treatment, and quarantine of infected individuals. Furthermore, the ability of our RDK to definitively diagnose AH1pdm virus infection, which cannot be done using conventional influenza A/B virus kits and is not be feasible by gene analyses such as real-time RT-PCR, would reduce the demand for vaccination against AH1pdm virus. Although we could test only one type of NP derived from H5N1 HPAI virus, which showed a negative result (data not shown), because of the limited availability of the viral strains, our RDKs showed sufficient specificity and sensitivity to detect AH1pdm virus. A large-scale multicenter evaluation of an RDK for AH1pdm virus to obtain approval from regulatory authorities is under way.

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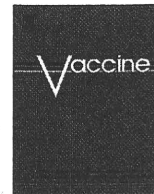

Safe and effective booster immunization using DTaP in teenagers

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ABSTRACT

The incidence of reported cases with pertussis has increased in young adults in Japan and the lack of additional booster immunizations containing pertussis components is suspected to be one of the causal reasons. Instead of DT immunization at 11–12 years of age, safety and immunogenicity were investigated using 0.2 ml and 0.5 ml of DTaP. 176 subjects in DTaP 0.5 ml, 178 in DTaP 0.2 ml, and 197 in DT 0.1 ml groups were enrolled in clinical trial. The relative risk of local reactions in the DTaP 0.2 ml group compared to the DT 0.1 ml group was 1.13 (95% CI: 0.97–1.30), and that of the DTaP 0.5 ml to the DT 0.1 ml group was 1.34 (95% CI: 1.18–1.53). The relative risks of local pain and heat were 1.62 (95% CI: 1.33–1.98) and 1.59 (95% CI: 1.19–2.13), respectively, in the DTaP 0.5 ml group compared to the DT 0.1 ml group. Seropositive rates against PT and FHA were 54% and 82% before immunization and increased to >95% for both after vaccination with no significant difference in GMT. Instead of the scheduled DT program, 0.2 ml of DTaP was acceptable and demonstrated efficient immunogenicity.

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1. Introduction

Pertussis is still a serious illness in young infants, causing whooping cough, apnea, cyanosis, chocking, and encephalopathy [1]. In Japan, whole cell pertussis vaccine was developed in 1950s and combined with diphtheria and tetanus toxoids (DTwP). DTwP became accepted, resulting in a reduction of reported cases of pertussis [2,3]. Approximately 10% of recipients experienced a febrile illness, with 50–60% showing redness and 20% induration [2]. In 1974–75, two accidental deaths after DTwP administration were reported and, thereafter, DTwP was discontinued for a while. It was re-introduced for children at 2 years of age, but the number of pertussis patients increased because of low vaccine coverage [2,3]. In 1981, a new type of acellular pertussis was developed, and combined vaccine (DTaP) was introduced into recommended immunization practice. Principally, two types of DTaP vaccines were developed: the B-type consisted of two major antigens, pertussis toxin (PT) and filamentous hemagglutinin (FHA) and the T-type contained pertactin and fimbrie besides PT and FHA [4–6]. Nationwide monitoring of clinical adverse events demonstrated low reactogenicity and sufficient antibody responses similar to natural infection. Since 1981, the number of pertussis patients decreased after the acceptance of DTaP. The incidence of pertussis in adults has been increasing gradually from 2002 in Japan, and several outbreaks on college campuses, and in high schools and offices have been reported [7,8]. In addition, the incidence in young infants less than 1 year of age increased as well as adult cases in 2009.

Pertussis is principally an infectious children's illness causing whooping and prolonged cough and the Advisory Committee on Immunization Practices (ACIP) recommended a 5-dose DTaP schedule, at ages 2, 4, 6, and 15–18 months and 4–6 years, instead of the previous DTwP in the US in 1997 [9]. In 1990s, the incidence of pertussis in older age increased in many countries because of waning immunity after primary childhood immunization and antigenic change of pertussis, and adolescent pertussis was identified as the source of transmission of pertussis to young infants through enhanced surveillance studies [10–16]. In 2005, tetanus toxoid, and a reduced concentration of diphtheria toxoid combined with reduced acellular pertussis (Tdap) vaccine was licensed, and the ACIP recommended that adolescents aged 11–18 years should receive a single dose of Tdap for booster immunization [17]. It was now recommended for all generations from 19 to 64 years [18].

It takes several years to obtain a license to introduce a new vaccine from foreign countries into Japan, even though Tdap is

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used worldwide. The immunization schedule in Japan incorporates no booster dose of pertussis components after the completion of the initial primary immunization (three times over 3 months of age and additional dose after approximately 12 months after the third dose), and vaccine containing pertussis components should be scheduled to cope with an outbreak of pertussis. In this study, safety and immunogenicity were investigated in clinical trials using 0.2 and 0.5 ml of DTaP at the age of 11–12 years, in comparison with 0.1 ml of DT.

2. Subjects and methods

2.1. Subjects

The study was conducted from September 2008 to August 2009, involving 29 pediatric outpatient clinics and departments of pediatrics of regional public and university hospitals. Subjects of this study included 555 children, 11–18 years of age, mostly 11–12 years of age, who had completed primary immunization of more than three doses of DTaP and had not undergone DT immunization. The study protocol was checked by the ethical committee of National Mie Hospital as a central organization and also checked by ethical committee of each hospital. Written informed consent was obtained from their parents or guardians. A total of 555 children were enrolled, but four children were excluded: three did not complete the primary immunization (one or two doses of DTaP), and one had already been immunized with DT. They were divided into two study groups: group 1 consisted of 266 subjects undergoing serological examination: 29 receiving 0.1 ml of DT, 119 for 0.2 ml of DTaP, and 118 for 0.5 ml of DTaP. Group 2 comprised 285 immunized without serological examinations, and totaling 551 subjects, with 197 receiving 0.1 ml DT, 178 for 0.2 ml of DTaP, and 176 for 0.5 ml of DTaP, were examined for safety. They are summarized in Fig. 1.

2.2. Vaccines

Five brands of DTaP were on the market in Japan, and the components of each antigen were different for each brand, as shown in Table 1. Subjects were allocated equally to each brand. The B-type (Biken and Kaketsu) vaccine consisted of PT and FHA and the T-type (Takeda, Denka, and Kitasato) contained other components, and the composition of pertussis antigens differed from the brands of DTaP available abroad [5,6,19]. The PT antigen contents varied from 3 to 23.5 μg/dose, and FHA from 23.5 to 51.5 μg/dose, but the amount of diphtheria and tetanus toxoids was 15 and 2.5 Lf/dose, respectively, without a difference among DTaP brands. 0.2 ml of DTaP contained 1.2–9.4 μg of PT, 9.4–20.6 μg of FHA, 6–6.6 Lf of diphtheria toxoid, and 1.0 Lf of tetanus toxoid. Antigen contents of FHA and diphtheria toxoid were slightly higher in 0.2 ml of DTaP than Tdap, Boostrix and Adacel (2.5–8 μg of PT, 5–8 μg of FHA, 2–2.5 Lf of diphtheria toxoid, and 5 Lf of tetanus toxoid) [17]. A 0.2-ml volume of DTaP contained similar amounts to Tdap. The antigen content of tetanus toxoid was lower in 0.2 ml of DTaP than Tdap available abroad, similar to 0.1 ml of DT.

2.3. Study design

The study was designed as a randomized open trial. Subjects were allocated randomly to DT 0.1 ml, DTaP 0.2 ml, and DTaP 0.5 ml groups. They were observed for 30 min for the appearance of anaphylaxis. To assess the safety afterwards, they were asked to check their body temperature and for adverse clinical events based on the healthcare diary every day for 7 days after immunization. In study group I, paired sera were obtained immediately before immunization and principally 4–6 weeks after immunization and kept at −20 °C. The paired sera were divided into two aliquots and transferred to the National Institute of Infectious Diseases, Department of Bacteriology II to examine antibodies against diphtheria and tetanus toxoids and to Kitasato-Otsuka Bio-Medical Assay Laboratories for the examination of pertussis antibodies (PT and FHA).

2.4. Serology

Antibodies against tetanus toxoid were determined with a KPA kit (Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) [20]. The kit comprised polypeptide artificial carrier particles stained with Reaction Blue solution, sensitized with highly purified tetanus toxoid (3000 Lf/mg PN), and provided in lyophilized form. The test was performed as instructed by the

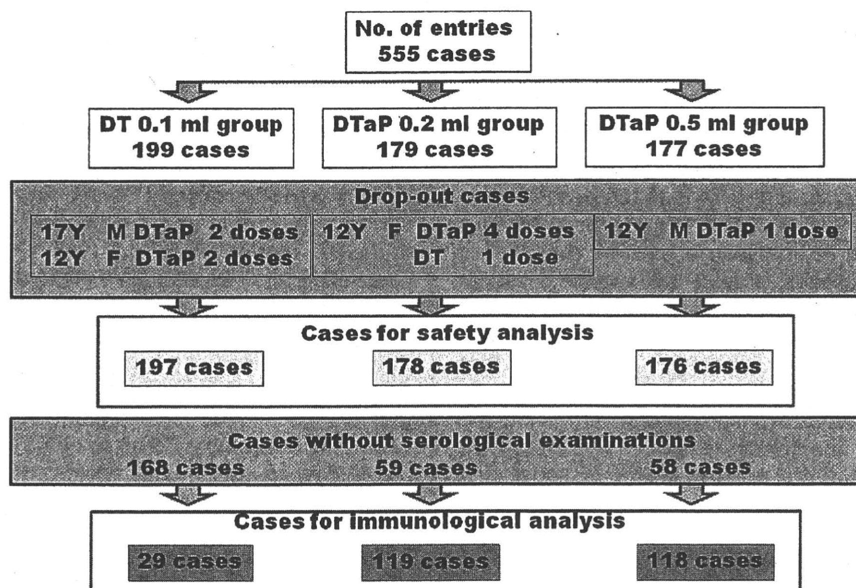

Fig. 1. Number of subjects in the study. A total of 555 subjects were enrolled, of whom four were excluded. Therefore, 551 subjects were evaluated regarding safety. Among the 551, 197 were immunized with 0.1 ml of DT, 178 with 0.2 ml of DTaP, and 176 with 0.5 ml of DTaP. Study group 1 consisted of 266 subjects for serological examination: 29 with 0.1 ml of DT, 119 with 0.2 ml of DTaP, and 118 with 0.5 ml of DTaP.

Table 1
Contents of PT, FHA, and diphtheria and tetanus toxoids.

DTaP/DT (manufacturers)	PT (μg)	FHA (μg)	Pertactin	Fimbrie	D (Lf)	T (Lf)
DTaP 0.5 ml (Kaketsu)	8	32			≦16.7	≒2.5
DTaP 0.5 ml (Biken)	23.5	23.5			≦15	≦2.5
DTaP 0.5 ml (Takeda)	3	34.5	7.5	1	≒15	≒2.5
DTaP 0.5 ml (Denka)	9	32	15	1	≦15	≦2.5
DTaP 0.5 ml (Kitasato)	6	51.5	5	1	≒15	≒2.5
Adacel (Aventis)	2.5	5	3		2	5
Boostrix (GSK)	8	8	2.5		2.5	5
DTaP 0.2 ml	1.2–9.4	9.4–20.6			6–6.6	1.0
DT 0.1 ml					3.2	0.7

Table 2
Background of the subjects.

	DTaP 0.2 ml (N = 178)	DTaP 0.5 ml (N = 176)	DT 0.1 ml (N = 197)	Total (N = 551)
Gender				
Male	93 (52.2%)	95 (54.0%)	113 (57.4%)	301 (54.6%)
Female	85 (47.8%)	81 (46.0%)	84 (42.6%)	250 (45.4%)
Age				
11 years	97 (54.5%)	95 (54.0%)	73 (37.1%)	265 (48.1%)
12 years	68 (38.2%)	68 (38.6%)	111 (56.3%)	247 (44.8%)
Others	13 (7.3%)	13 (7.4%)	13 (6.6%)	39 (7.1%)
Mean age ± SD	11.6 ± 0.8	11.6 ± 0.8	11.8 ± 0.8	11.6 ± 0.8
Median age	11.0	11.0	12.0	12.0
Range (min–max)	(11–15)	(11–15)	(11–17)	(11–17)
DPT history				
I-1	178 (100.0%)	176 (100.0%)	197 (100.0%)	551 (100.0%)
I-2	178 (100.0%)	176 (100.0%)	197 (100.0%)	551 (100.0%)
I-3	172 (96.6%)	172 (97.7%)	193 (98.0%)	537 (97.5%)
I-boost	172 (96.6%)	168 (95.5%)	191 (97.0%)	531 (96.4%)

manufacturers. Antibodies against diphtheria toxoid were examined using the micro cell-culture method with Vero cells, and diphtheria antitoxin titers were expressed as international units (IU)/ml [21]. Antibodies against PT and FHA were examined using enzyme-linked immunosorbent assay (EIA) kits (Wako Chemicals, Japan) as instructed by the manufacturers. Positive levels were defined as ≥0.1 IU/ml for antibodies against diphtheria toxoid, ≥0.01 IU/ml for those against tetanus toxoid, and ≥10 EU/ml for those against PT and FHA [22,23].

2.5. Statistical analysis

The sero-positivity rate and the incidence of solicited adverse events (fever as systemic reaction, and redness, swelling, pain, heat, and itching as local reactions) were compared by using Fisher's Extraction test. Geometric mean titers (GMTs) of antibodies before and after immunization were compared by converting to a logarithmic scale using Wilcoxon rank test. The *t* student Welch method was employed to evaluate significance and the significant level was set at $p < 0.05$.

3. Results

3.1. Background of the subjects

The subjects included 555 children aged 11–18 years of age, as shown in Fig. 1. A total of 555 subjects were enrolled, but four were excluded. Therefore, 551 subjects were evaluated for safety. Among the 551, 197 were immunized with 0.1 ml of DT, 178 with 0.2 ml of DTaP, and 176 with 0.5 ml of DTaP. The backgrounds of the subjects are shown in Table 2. A total of 301 (54.6%) were male, and the gender ratio was similar among the three groups with no significant differences in ages, which ranged from 11 to 17 years. They had all completed their primary immunizations (three or four doses of DTaP), confirmed by checking their immunization records.

3.2. Incidence of adverse events

The incidences of adverse events are summarized in Table 3. Febrile reactions were noted in 8 (4.1%) of 197 in the DT 0.1 ml group, 7 (3.9%) of 178 in the DTaP 0.2 ml group, and 7 (4.0%) of 176 in the DTaP 0.5 ml group, and the relative risks in DTaP

Table 3
Incidence of clinical adverse events.

Adverse events	DTaP 0.2 ml (1)	DTaP 0.5 ml (2)	DT 0.1 ml (3)	Risk ratio (95% CI)		
	(N = 178)	(N = 176)	(N = 197)	(2) vs. (1)	(1) vs. (3)	(2) vs. (3)
Fever	7 (3.9%)	7 (4.0%)	8 (4.1%)	1.01 (0.36,2.82)	0.97 (0.36,2.62)	0.98 (0.36,2.65)
Local reactions	123 (69.1%)	145 (82.4%)	121 (61.4%)	1.19 (1.06,1.34)	1.13 (0.97,1.30)	1.34 (1.18,1.53)
Redness	95 (53.4%)	109 (61.9%)	92 (46.7%)	1.16 (0.97,1.39)	1.14 (0.93,1.40)	1.33 (1.10,1.60)
Swelling	90 (50.6%)	95 (54.0%)	76 (38.6%)	1.07 (0.87,1.30)	1.31 (1.04,1.65)	1.40 (1.12,1.75)
Pain	83 (46.6%)	116 (65.9%)	80 (40.6%)	1.41 (1.17,1.71)	1.15 (0.91,1.45)	1.62 (1.33,1.98)
Heat	50 (28.1%)	74 (42.0%)	52 (26.4%)	1.50 (1.12,2.00)	1.06 (0.76,1.48)	1.59 (1.19,2.13)
Itching	81 (45.5%)	83 (47.2%)	75 (38.1%)	1.02 (0.82,1.28)	1.21 (0.95,1.54)	1.24 (0.98,1.57)

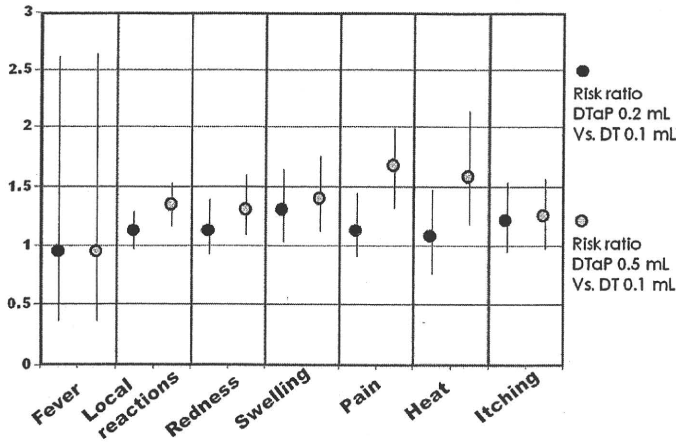

Fig. 2. Summary of the risk ratio regarding the incidence of adverse reactions. The relative risks of the incidence of adverse reactions after immunization with 0.2 ml (●) and 0.5 ml (◉) of DTaP in comparison with those observed after immunization with 0.1 ml of DT are summarized. Vertical lines represent 95% CI.

0.2 ml and DTaP 0.5 ml groups were 0.97 and 0.98, respectively, in comparison with that observed in the DT 0.1 ml group. The relative risk of local reactions after immunization with DTaP at 0.2 ml was 1.13 (95% CI: 0.97–1.30) in comparison with the incidence after immunization with DT at 0.1 ml, and that of the DTaP 0.5 ml compared to the DT 0.1 ml group was 1.34 (95% CI: 1.18–1.53). Relative risks of redness, swelling, local pain, heat, and itching in the DTaP 0.2 ml group compared to the DT 0.1 ml group were 1.14 (95% CI: 0.93–1.40), 1.31 (95% CI: 1.04–1.65), 1.15 (95% CI: 0.91–1.45), 1.06 (95% CI: 0.76–1.48), and 1.21 (95% CI: 0.95–1.54), respectively. However, the relative risks of redness, swelling, local pain, heat, and itching in the DTaP 0.5 ml group compared to the DT 0.1 ml group were 1.33 (95% CI: 1.10–1.60), 1.40 (95% CI: 1.12–1.75), 1.62 (95% CI: 1.33–1.98), 1.59 (95% CI: 1.19–2.13), and 1.24 (95% CI: 0.98–1.57), respectively. The relative risks of the adverse reactions after immunization in the DTaP 0.2 ml and 0.5 ml groups in comparison with those observed after immunization in the DT 0.1 ml group are summarized in Fig. 2. Thus, the incidence of local reactions after immunization with 0.2 ml of DTaP was similar to that observed after immunization with 0.1 ml of DT, but those observed after immunization with 0.5 ml of DTaP were higher than after immunization with 0.1 ml of DT, notably regarding the incidences of local pain and heat, demonstrating the relative risks: 1.62 (95% CI: 1.33–1.98) and 1.59 (95% CI: 1.19–2.13), respectively.

3.3. *Onset of adverse reactions*

The immunization day was defined as day 0. The onset of adverse reactions was examined, and the results are shown in Fig. 3. Febrile reactions were noted from days 0 to 7 without any case accumulation, but the incidence of local reactions peaked on days 1 and 2. Systemic adverse events were reported sporadically: headache in 25 (9 in DT 0.1 ml group, 9 in DTaP 0.2 ml group, and 7 in DTaP 0.5 ml group), fatigue in 11 (3 in DT 0.1 ml group, 4 in DTaP 0.2 ml group, and 4 in DTaP 0.5 ml group), rhinorrhea in 10 (1 in DT 0.1 ml, 2 in DTaP 0.2 ml, and 7 in DTaP 0.5 ml group), sore throat in 8, cough in 7, and nasal obstruction in 7. Three subjects with urticaria eruption were reported: two on day 0 (one for each DT 0.1 ml and DTaP 0.5 ml group) and one on day 1 in DTaP 0.5 ml group. Generalized eruption was reported on day 1 in DTaP 0.5 ml group. The relative risk of local reactions on day 0 after immunization with 0.2 ml of DTaP compared to that observed after 0.1 ml of DT was 1.08 (95% CI: 0.74–1.58), 1.18 (95% CI: 0.96–1.44) on day 1, 1.09 (95% CI: 0.91–1.30) on day 2, 1.19 (95% CI: 0.97–1.47) on day 3, 1.3 (95% CI: 0.99–1.71) on day 4, 1.56 (95% CI: 1.09–2.23) on day 5, 1.42 (95% CI: 0.87–2.29) on day 6, and 1.54 (95% CI: 0.87–2.72) on day 7. The incidence of local reaction for each day after immunization with 0.2 ml of DTaP was similar to that observed after 0.1 ml of DT. The incidence of local reactions after immunization with 0.5 ml of DTaP was higher than that observed in the DT 0.1 ml group, especially on days 1 and 2, with a relative risk of 1.61 (95% CI: 1.35–1.92) on day 1, and 1.33 (95% CI: 1.13–1.92) on day 2. Most local adverse reactions appeared on day 1 and continued for 3–4 days, but those observed in the DTaP 0.5 ml group became prolonged, showing a relative risk of 2.15 (95% CI: 1.39–3.33) on day 6.

In this study, the extents of redness and swelling were monitored when they appeared and the degree of adverse reactions was evaluated (Fig. 4). There was no significant difference in the incidence of redness and swelling of <2.0 cm and 2–5 cm among the

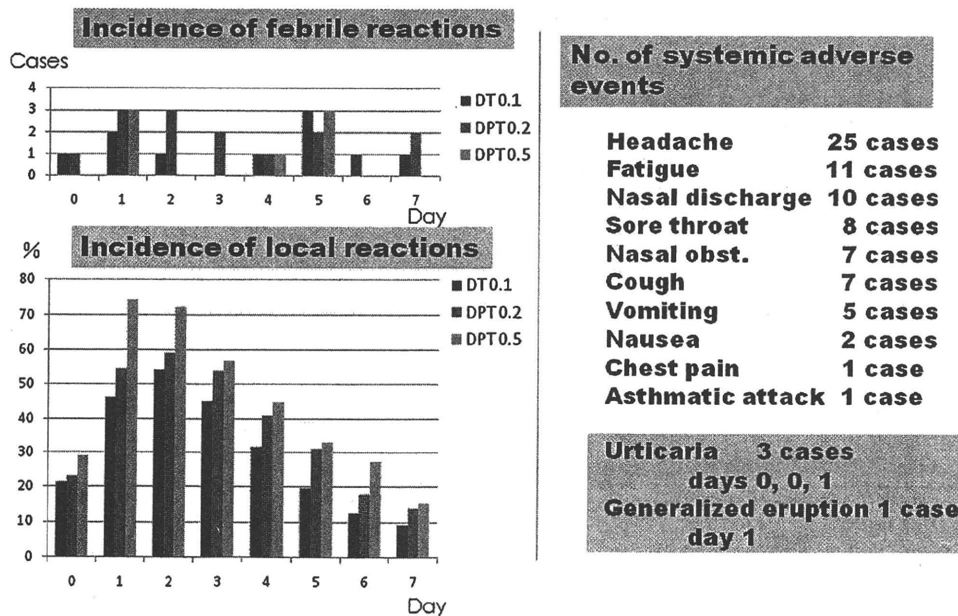

Fig. 3. Onset of febrile and local reactions within 7 days after immunization and the no. of cases with systemic adverse events.