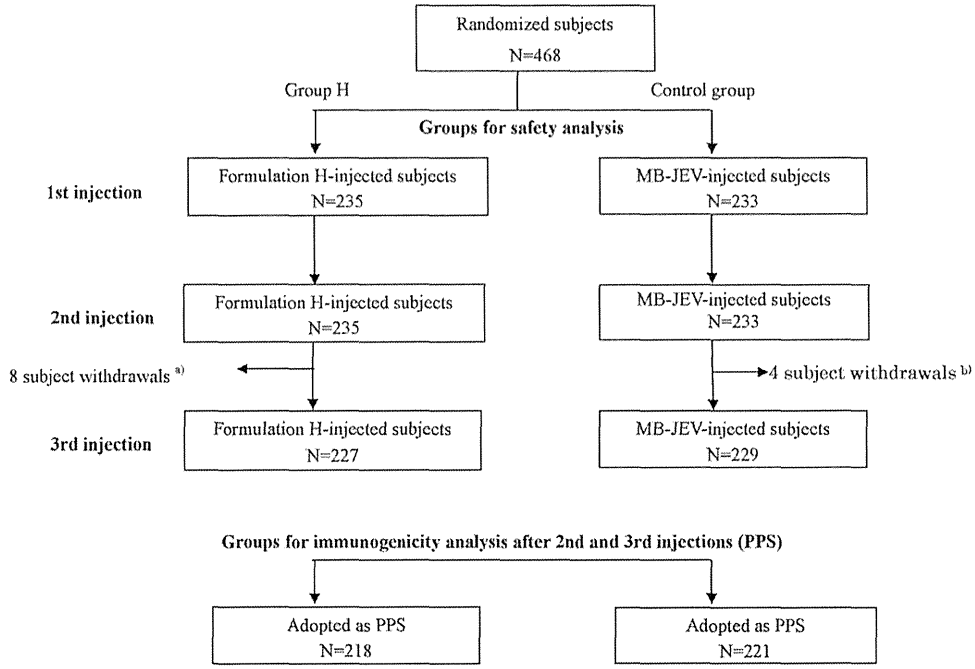

FIG 1 Disposition of the subjects in trial 1. [a], 8 subjects withdrew: 2 because of cancellations of the trial by parent or guardian of the subjects, 2 for the occurrence of adverse events, 1 because of change of residence, and 3 because of judgments by the primary investigator. [b], 4 subjects withdrew: 3 because of cancellations of the trial by parent or guardian of the subjects and 1 because of a positive antibody titer before injection.

mamoto, Japan), approved in 2011. In mice, JeBIK-V showed superior neutralizing antibody titers compared with those of MB-JEV (8). The safety and immunogenicity of JeBIK-V were also shown in children (9). However, as MB-JEV was not used as a comparator in the study, the comparisons of the immunogenicity and safety between the CC-JEV and MB-JEV were not performed simultaneously. The immunogenicity and safety of a CC-JEV vaccine, Ixiaro (Intercell Biomedical, Livingston, United Kingdom), assessed in clinical phase III trial, were also reported (10). This vaccine is a purified inactivated aluminum-adjuvanted JE vaccine

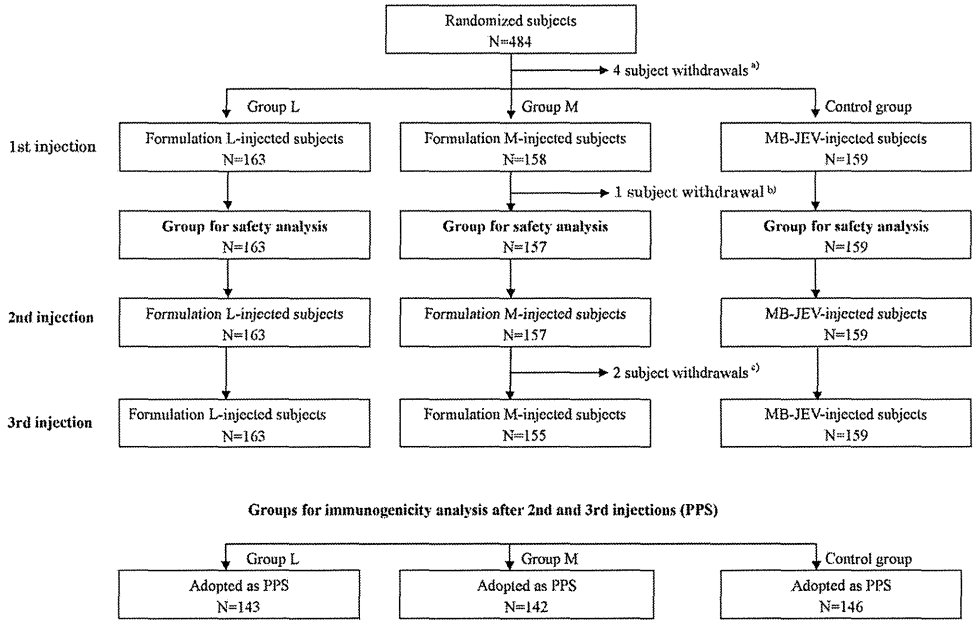

FIG 2 Disposition of the subjects in trial 2. [a], of the 484 randomized subjects, 4 subjects withdrew: 2 because of cancellations of the trial by parent or guardian of the subjects and 2 because of judgments by the investigator. [b], of the 158 formulation M-injected subjects, 1 subject withdrew because a drug with a nonallocated number was injected after the 2nd injection. [c], of the 157 formulation M-injected subjects, 2 withdrew: 1 because of cancellation of the trial by parent or guardian of the subject and 1 because of a positive antibody titer before injection.

TABLE 1 Background data of subjects in trial 1

Background factor	Group H (formulation H) (n = 218)[a] Value	95% CI (%)[b]	Control group (MB-JEV) (n = 221)[a] Value	95% CI (%)
Gender (n [%])				
Male	107 (49.1)	42.3–55.9	115 (52.0)	45.2–58.8
Female	111 (50.9)	44.1–57.7	106 (48.0)	41.2–54.8
Age (mean [SD]) (mo)	36.9 (13.4)	NC[c]	36.7 (15.5)	NC
Body wt (mean [SD]) (kg)	13. 9 (2.7)	NC	13.8 (3.3)	NC

[a] Injected vaccine.
[b] CI, confidence interval.
[c] NC, not calculated.

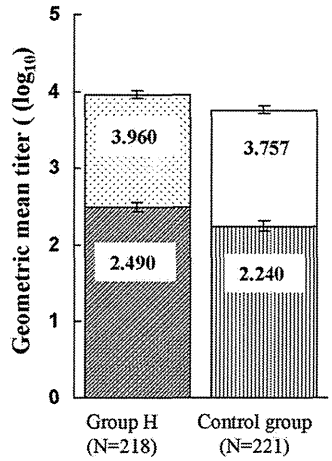

FIG 3 GMTs after the 2nd and 3rd injections in trial 1. Two groups of 235 and 233 subjects were injected subcutaneously with a series of two doses of 0.5 ml of formulation H or MB-JEV, respectively, at an interval of 1 to 4 weeks, and an additional injection was given 6 to 15 months after the 2nd injection. The GMTs after the 2nd and 3rd injections in trial 1 are shown after the 2nd (shaded area) and 3rd (dotted area) injections in group H and after the 2nd (striped area) and 3rd (white area) injections in the control group. The error bars represent 95% confidence intervals.

produced using the SA14-14-2 virus strain, which is used for persons ≥17 years of age in North America, Europe, and Australia (under the label Jespect) (11). In all these trials, however, a study on a direct comparison of the immunogenicity and safety between CC-JEV and MB-JEV produced using the Beijing-1 strain has not been reported so far. We report here the results of two series of phase III trials (trials 1 and 2) conducted in children simultaneously using CC-JEV (Encevac) and MB-JEV, produced using the same Beijing-1 strain (these studies have been registered at the JapicCTI under registration no. JapicCTI-132063 and JapicCTI-080586 for trials 1 and 2, respectively).

MATERIALS AND METHODS

Vaccines. The CC-JEV was manufactured according to the method described by Sugawara et al. (6). Briefly, Vero cells were passaged in a culture medium containing 2% fetal bovine serum and microcarriers. When cell numbers reached approximately 2×10^6 cells/ml, cells cultured by microcarriers were isolated and inoculated with the Beijing-1 strain. A serum-free medium was added after virus adsorption, and thereafter, the cells were cultured for 4 days at 37°C. The culture supernatant was then harvested and concentrated by ultrafiltration. After inactivation with formalin, the vaccine antigens were purified by sucrose density gradient centrifugation, in addition to the manufacturing method used for the production of the MB-JEV. For the production of the CC-JEV, additional affinity column chromatography using Cellufine sulfate (JNC Corporation, Tokyo, Japan) was added to achieve further purification by removing residual host cell-derived proteins and DNAs from the final bulk vaccine. Two series of phase III clinical trials, trials 1 and 2, were conducted using the CC-JEV as a test vaccine. In trial 1, formulation H, which was a liquid-type vaccine containing 17 μg/dose of CC-JEV in 0.5 ml, was used. In trial 2, two freeze-dried types of the CC-JEV vaccine instead of the liquid-type vaccine were used to increase the stability; these were formulations L and M, containing 4 μg/dose and 8 μg/dose of CC-JEV in 0.5 ml, respectively. The MB-JEV, used as a comparator vaccine in both trials, was manufactured by the Chemo-Sero-Therapeutic Research Institute (Kaketsuken), Kumamoto, Japan, as follows: the Beijing-1 strain was inoculated into the brains of 4-week-old ddy strain mice. The brains of the mice showing symptoms of encephalitis were collected to prepare a brain emulsion. After centrifugation, the supernatant of the emulsion was treated with protamine sulfate to remove the mouse brain-derived materials. The virus fluid was recovered from the supernatant, and the supernatant was subjected to the sucrose density gradient centrifugation. After centrifugation, the virus particle faction was collected and then treated with formalin for inactivation in order to prepare an inactivated purified virus preparation. Finally, the total amount of protein of the MB-JEV was adjusted to 34 μg/ml. In both trials, the liquid MB-JVE containing 17 μg/dose of vaccine antigen in 0.5 ml was used as a comparator vaccine. The vaccine was administered subcutaneously.

Vaccination design. Two series of phase III clinical trials, trials 1 and 2, were conducted as double-blinded randomized parallel-group studies in multiple facilities. The subjects were children 6 to 89 months of age without a history of JE vaccine or of JEV infection. The main exclusion criteria were: (i) subjects with acute serious diseases, (ii) subjects who clearly presented anaphylaxis to vaccine components or excipients, (iii) subjects who had received a blood transfusion or administration of gamma-globulin preparation within 3 months before the start of the trial or who had received a massive dose therapy of gamma-globulin preparation (≥200 mg/kg of body weight) within 6 months before the start of the trial, and (iv) subjects for whom enrollment in the trial was judged by the principal investigator or subinvestigators as not appropriate. Written informed consent was obtained from a parent or guardian of each subject before trial enrollment. Prior to the conduction of the trials, the trial-related forms, such as the trial protocols, were investigated by the institu-

TABLE 2 Seroconversion rates of neutralizing antibody after the 2nd and 3rd injections in trial 1

Time	Group H (formulation H) % (no. of positive conversions/no. of analyzed subjects)	95% CI[a]	Control group (MB-JEV) % (no. of positive conversions/no. of analyzed subjects)	95% CI	Noninferiority test
After 2nd injection	100.0 (218/218)	98.3–100.0	99.5 (220/221)	97.5–100.0	NC[b]
After 3rd injection	100.0 (218/218)	98.3–100.0	100.0 (221/221)	98.3–100.0	$P < 0.001$

[a] CI, confidence interval.
[b] NC, not calculated.

TABLE 3 Vaccine-related adverse events over three injections (≥5%) in trial 1

Reaction	Group H (formulation H) (n = 235)			Control group (MB-JEV) (n = 233)		
	n	%	95% CI[a]	n	%	95% CI
At injection site						
Erythema	22	9.4	6.0–13.8	13	5.6	3.0–9.4
Swelling	13	5.5	3.0–9.3	6	2.6	1.0–5.5
Systemic						
Fever	18	7.7	4.6–11.8	23	9.9	6.4–14.4

[a] CI, confidence interval.

tional review boards/independent ethics committees of the facilities conducting the trials or of the base hospital of the relevant network. The trials were conducted in compliance with the ethical doctrine based on the Helsinki Declaration, the good clinical practice regulations, and other relevant regulations.

Trial 1. Trial 1 was conducted from February 2003 to August 2004. A flowchart depicting the dispositions of the subjects in trial 1 is shown in Fig. 1. After obtaining informed consent, 468 qualified subjects were recruited for trial 1; among them, 235 and 233 subjects were assigned to two groups and were injected subcutaneously with a series of 2 doses of 0.5 ml of formulation H or the MB-JEV, respectively, at an interval of 1 to 4 weeks, and an additional injection was given 6 to 15 months after the 2nd injection. For the children <3 years of age, doses of 0.25 ml were given using the same vaccination schedule.

Trial 2. Trial 2 was conducted from June 2008 to May 2009. A flowchart depicting the dispositions of the subjects in trial 2 is shown in Fig. 2. After obtaining informed consent, 480 qualified subjects were recruited for trial 2; among them, 163, 158, and 159 subjects were assigned to three groups, and were injected subcutaneously with a dose of 0.5 ml of formulation M, formulation L, or the MB-JEV, respectively, at an interval of 2 to 4 weeks, and an additional injection was given 1 to 15 months after the 2nd injection. For the children <3 years of age, doses of 0.25 ml were given using the same vaccination schedule.

Randomization. In both trials, randomizations were performed according to a computer-generated algorithm. Trial 1 was performed with a permuted-block design. Trial 2 was performed with a stratified randomization as a stratified factor of the vaccine volumes. The eligible subjects were randomly assigned in a 1:1 ratio for trial 1 and a 1:1:1 ratio for trial 2. In both trials, the statistician generating the randomization algorithm was not involved in determining the eligibility, vaccination course, or determining outcomes of the subjects.

Blinding. In trial 1, the test vaccine and the comparator vaccine were liquid-type vaccines with an identical appearance. However, in trial 2, the test vaccine was a freeze-dried type, while the comparator vaccine was a liquid type. Therefore, to ensure blinding, an investigational drug coordinator who prepared the investigational drug for a doctor and cleared it off after the inoculation instead of the doctor was specially assigned at each hospital.

Safety analysis. In trial 1, a parent or guardian of each subject recorded the presence or absence of adverse events in a health diary every day for 7 days after each inoculation. In trial 2, a parent or guardian of each subject recorded the presence or absence of adverse events in a health diary every day for 13 days after each inoculation. Adverse events were recorded until 27 days after inoculation when they were recognized. Regarding local reactions, including erythema or swelling at the injection site, a reaction of ≥2 cm in diameter was recorded as an adverse event in trial 1, and any reaction regardless of its size was recorded as an adverse event in trial 2. A fever of ≥37.5°C was recorded as an adverse event in both trials. In trial 1, the severity of adverse events was judged by the principal investigator or a subinvestigator and was classified into three levels, mild, moderate, and severe, considered for their interference with normal daily activities. Similarly, in trial 2, the criteria were defined in terms of grades 1 to 4, according to severity. Adverse events for which a possible relationship with one of the test vaccines could not be denied were judged as vaccine-related adverse events.

Immunological analysis. In trial 1, blood serum samples for the measurement of antibody titers were obtained from each subject before the 1st injection and 2 to 6 weeks after the 2nd and 3rd injections. In trial 2, serum samples were obtained from each subject before the 1st injection and 4 to 6 weeks after the 2nd and 3rd injections. The sera were stored at −20°C until the time of measurement. Neutralizing antibodies against a JE virus strain, Beijing-1, were measured by a 50% plaque reduction method using Vero cells and calculated using the 3 points least-squares regression method (3LSRM) (12). Briefly, Vero cells grown in six-well plates (Costar six-well cell culture cluster, flat bottom, with lid, catalog no. 3506; Corning Incorporated, Corning, NY, USA) were used. Serial dilutions of the serum samples were carried out routinely to 1:10, 1:40, 1:160, 1:640, 1:2,560, and 1:10,240, and the challenge virus (Beijing-1 strain) was also diluted to give 100 plaques per well. One more dilution was added and the rest repeated if there was not a 50% plaque reduction. The same volume of diluted serum samples and virus were mixed and added to the cell-seeded wells in triplicate. The control virus was added to 12 wells. The plates were then incubated for 90 min at 37°C, and overlay medium was added. Following 5 days at 37°C incubation, 10% formalin was added, followed by methylene blue tetrahydrate to stain the virus plaques. If the average value of the number of plaques in ≥10 control wells was between 50 and 150, the assay was accepted. The neutralizing antibody titer was expressed as the reciprocal of the dilution of serum that caused a 50% reduction of plaque formation compared to the plaque number of the diluted challenge virus in the absence of antiserum. Antibody positive was defined as the neutralizing antibody titer being ≥1:10 (13).

Statistical analysis of antibody titers. In both trials, the primary endpoint was the seroconversion rate after the 3rd injection based on the

TABLE 4 Background data of subjects in trial 2

Background factor	Group (injected vaccine type) (n)					
	L (formulation L) (143)		M (formulation M) (142)		Control (MB-JEV) (146)	
	Value	95% CI[a]	Value	95% CI	Value	95% CI
Gender (n [%])						
Male	77 (53.8)	45.3–62.2	73 (51.4)	42.9–59.9	68 (46.6)	38.3–55.0
Female	66 (46.2)	37.8–54.7	69 (48.6)	40.1–57.1	78 (53.4)	45.0–61.7
Age (mean [SD]) (mo)	48.5 (18.2)	NC[b]	48.5 (16.1)	NC	47.7 (17.3)	NC
Body wt (mean [SD]) (kg)	15.7 (3.9)	NC	15.5 (3.1)	NC	15.4 (3.4)	NC

[a] CI, confidence interval.
[b] NC, not calculated.

TABLE 5 Seroconversion rates of neutralizing antibody after the 2nd and 3rd injections in trial 2

Time	Group L (formulation L)		Group M (formulation M)		Control group (MB-JEV)		Noninferiority test
	% (no. of positive conversions/no. of analyzed subjects)	95% CI[a]	% (no. of positive conversions/no. of analyzed subjects)	95% CI	% (no. of positive conversions/no. of analyzed subjects)	95% CI	
After 2nd injection	100.0 (143/143)	97.5–100.0	100.0 (141/141)	97.4–100.0	94.5 (138/146)	89.5–97.6	NC[b]
After 3rd injection	100.0 (143/143)	97.5–100.0	100.0 (140/140)	97.4–100.0	100.0 (146/146)	97.5–100.0	$P < 0.001$

[a] CI, confidence interval.
[b] NC, not calculated.

neutralizing antibody titer. The secondary endpoints were the seroconversion rate after the 2nd injection and the geometric mean antibody titers (GMTs) after the 2nd and 3rd injections. The per-protocol set (PPS) was used to represent the immunogenicity population. Statistical analyses were performed using SAS version 8.2 for trial 1 and SAS version 9.1 for trial 2.

Trial 1. In trial 1, the noninferiority in the seroconversion rate after the 3rd injection for the formulation H-injected group (group H) against the MB-JEV group (control group) was statistically analyzed by the method of Dunnett and Gent (14). A sample size of 100 subjects per group was calculated to verify the noninferiority in the seroconversion rate (assumed to have an expected seroconversion rate of 95%) in each group ($\alpha = 0.025$ [1-tailed test], $\beta = 0.10$, 90% power, and noninferiority limit $\delta = 10\%$). Assuming that the seropositive rate was 45% before the trial and the withdrawal rate was 10%, 204 subjects in each group, for a total of 408 subjects, were required.

Trial 2. In trial 2, the noninferiority in the seroconversion rates after the 3rd injection for the formulation L-injected group (group L) and the formulation M-injected group (group M) against the control group were statistically analyzed by the Farrington-Manning method (15). A sample size of 81 subjects for each group was calculated to verify noninferiority (assumed to have an expected seroconversion rate of 98% against JE virus after the 3rd injection in both groups ($\alpha = 0.025$ [1-tailed test], $\beta = 0.10$, 90% power, and noninferiority limit $\delta = 10\%$). Assuming that the seropositive rate was 10% before the trial and that the withdrawal rate was 10%, 100 subjects in each group, for a total 300 subjects, were required.

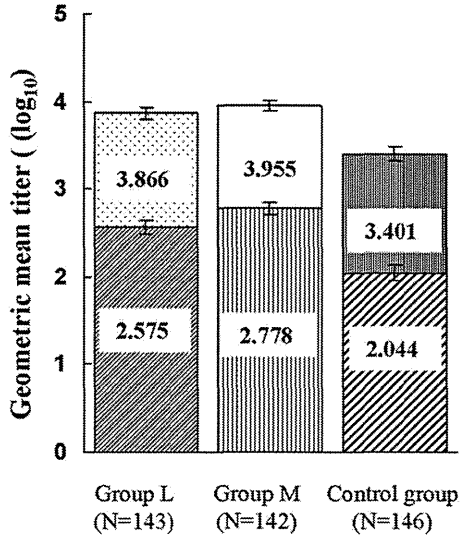

FIG 4 GMTs after the 2nd and 3rd injections in trial 2. Three groups of 158, 163, and 159 subjects were injected subcutaneously with a 0.5-ml dose of formulation L, formulation M, and MB-JEV, respectively, at an interval of 2 to 4 weeks, and an additional injection was given 1 to 15 months after the 2nd injection. The GMTs after the 2nd and 3rd injections in trial 2 are shown after the 2nd (shaded area) and 3rd (dotted area) injections in group L, after the 2nd (striped area) and 3rd (white area) injections in group M, and after the 2nd (shaded striped area) and 3rd (striped area) injections in the control group. The error bars represent 95% confidence intervals.

RESULTS

Trial 1. The main purpose of trial 1 was to verify the noninferiority in the seroconversion rates of group H to the control group after the 3rd injection. The subjects were injected with either formulation H or the MB-JEV. Among the 468 subjects enrolled in this trial, 235 subjects and 233 subjects were assigned randomly to either group H or the control group, respectively, and all of them were included in the safety population, while 439 subjects were included in the PPS immunogenicity population (group H, 218; control group, 221) (Fig. 1). The main protocol deviations were from specified inoculation and blood collection time points; subjects with deviations were excluded from the PPS. In trial 1, the seropositive rate was much lower than our *a priori* assumption of 45% based on the results of the National Epidemiological Surveillance of Vaccine-Preventable Diseases by the Ministry of Health, Labor, and Welfare. Although the number of subjects was greater than the planned sample size, the objective of the trial was achieved. The background data of the subjects are shown in Table 1. No statistically significant differences were observed in the baseline factors between the two groups.

Immunogenicity in trial 1. The seroconversion rates after the 3rd injection were 100% in both groups. The results analyzed by the Dunnett and Gent test verified that group H was not inferior to the control group in terms of the seroconversion rate, which met the primary endpoint. On the other hand, the seroconversion rate of group H after the 2nd injection was 100%, while that of the control group was 99.5% (Table 2). The GMTs of group H after the 2nd and 3rd injections were statistically higher than those of the control group (Fig. 3). The differences in the GMTs of subjects in group H and the control group were assessed using a *t* test ($P < 0.001$).

Safety in trial 1. In trial 1, vaccine-related adverse events with an incidence of ≥5% are listed in Table 3. The incidences of vaccine-related adverse events, such as injection site erythema and injection site swelling, were higher in group H than those of the control group; however, there were no statistically significant differences between the two groups. In group H, erythema exsudativum multiforme (a series of diseases in which erosion develops in the mucosa, such as the lips, oral cavity, and eyes, or multiple erythema or erosion lesions develop on the skin of the entire body) occurred in one subject 9 days after the 2nd injection with formulation H. The subject had recovered well 10 days after hospitaliza-

TABLE 6 GMTs after the 3rd injection in four patterns different in vaccine volume in trial 2

Vaccine volumes at 1st/2nd/3rd injections (ml)	Group (injected vaccine type) (*n*)[a]					
	L (formulation L) (143)		M (formulation M) (142)		Control (MB-JEV) (146)	
	No. receiving dose	GMT (log_{10}) (95% CI)	No. receiving dose	GMT (log_{10}) (95% CI)	No. receiving dose	GMT (log_{10}) (95% CI)
0.25/0.25/0.25	21	3.830 (3.612–4.047)	15	3.793 (3.518–4.069)	19	3.211 (2.944–3.478)
0.25/0.25/0.5	10	3.939 (3.744–4.134)	10	3.999 (3.801–4.197)	9	3.452 (3.109–3.796)
0.25/0.5/0.5	0	NA	2	4.535 (NA)	0	NA
0.5/0.5/0.5	112	3.866 (3.795–3.937)	113	3.962 (3.898–4.026)	118	3.428 (3.348–3.508)

[a] GMT, geometric mean titer; CI, confidence interval; NA, not applicable.

tion. The doctor who took care of this subject commented that a relationship between this case and formulation H could not be completely denied, although a viral infection was suspected because fever after pharyngeal erythema was observed. Based on this doctor's comment, this case was judged as a serious vaccine-related adverse event.

The immunogenicity of formulation H was greater than that of the MB-JEV. However, as the incidence of injection site vaccine-related adverse events for formulation H was higher than that of the MB-JEV and a serious vaccine-related adverse event occurred after the injection of formulation H, we conducted an additional trial, trial 2, to adjust the immunogenicity of the CC-JEV to match that of the MB-JEV.

Trial 2. The main purpose of trial 2 was to verify the noninferiority in the seroconversion rates of group L and group M to the control group after the 3rd injection. The subjects were injected with either formulation L, formulation M, or the MB-JEV. Among the 484 subjects enrolled in this trial, the subjects were assigned randomly into group L (n = 163), group M (n = 158), or the control group (n = 159), and group L (n = 163), group M (n = 157; one subject withdrew), or the control group (n = 159) were included in the safety test population. For the PPS immunogenicity test population, the numbers of included subjects were as follows: group L, 143; group M, 142; and the control group, 146 (Fig. 2). The main protocol deviations were the use of prohibited medications and deviations from the specified enrollment procedures; subjects with deviations were excluded from the PPS. In trial 2, the seropositive rate was much lower than our *a priori* assumption of 10%, which was made based on the results of trial 1. Although the number of subjects was greater than the planned sample size, the objective of the trial was achieved. The background data of the subjects are shown in Table 4. No statistically significant differences were observed in the baseline factors among the three groups.

Immunogenicity in trial 2. The seroconversion rates of all groups after the 3rd injection were 100%. The results analyzed by the Farrington-Manning test verified that groups L and M were not inferior to the control group in terms of the seroconversion rate, which met the primary endpoint. On the other hand, while the seroconversion rates of groups L and M after the 2nd injection were 100%, the seroconversion rate of the control group was 94.5% (Table 5). The GMTs of groups L and M after the 2nd and 3rd injections were statistically higher than those of the control group (Fig. 4). The differences in the GMTs of subjects in group L, group M, and the control group were assessed using a t test ($P <$ 0.001). These results showed that the neutralizing antibody titer

TABLE 7 Vaccine-related adverse events over three injections (≥5%) in trial 2

Reaction	Group (injected vaccine type) (*n*)								
	L (formulation L) (163)			M (formulation M) (157)			Control (MB-JEV) (159)		
	No. with adverse reaction	%	95% CI[a]	No. with adverse reaction	%	95% CI	No. with adverse reaction	%	95% CI
Local reactions									
Erythema	27	16.6	11.2–23.2	39	24.8	18.3–32.4	33	20.8	14.7–27.9
Swelling	11	6.7	3.4–11.8	13	8.3	4.5–13.7	13	8.2	4.4–13.6
Induration	3	1.8	0.4–5.3	8	5.1	2.2–9.8	4	2.5	0.7–6.3
Itching	1	0.6	0.0–3.4	2	1.3	0.2–4.5	13	8.2	4.4–13.6
Systemic reactions									
Fever	35	21.5	15.4–28.6	44	28.0	21.2–35.7	23	14.5	9.4–20.9
Grade ≥3 (≥39.0°C)	3	1.8	0.4–5.3	8	5.1	2.2–9.8	2	1.3	0.2–4.5
Coughing	13	8.0	4.3–13.3	9	5.7	2.7–10.6	11	6.9	3.5–12.0
Nasal drainage	11	6.7	3.4–11.8	11	7.0	3.5–12.2	8	5.0	2.2–9.7
Rash	9	5.5	2.6–10.2	4	2.5	0.7–6.4	4	2.5	0.7–6.3
Diarrhea	6	3.7	1.4–7.8	6	3.8	1.4–8.1	8	5.0	2.2–9.7
Grade ≥3[b]	0	0	0.0–2.2	0	0	0.0–2.3	1	0.6	0.0–3.5
Headache	4	2.5	0.7–6.2	4	2.5	0.7–6.4	8	5.0	2.2–9.7

[a] CI, confidence interval.

[b] Diarrhea grade of ≥3 defined as an increase in stool frequency of ≥9 times/day.

TABLE 8 Vaccine-related adverse event of fever over three injections in trial 2

Fever grade[a]	Group (injected vaccine type) (*n*)					
	L (formulation L) (163)		M (formulation M) (157)		Control (MB-JEV) (159)	
	No. with fever (%)	95% CI[b]	No. with fever (%)	95% CI	No. with fever (%)	95% CI
Total	35 (21.5)	15.4–28.6	44 (28.0)	21.2–35.7	23 (14.5)	9.4–20.9
1	22 (13.5)	8.7–19.7	20 (12.7)	8.0–19.0	14 (8.8)	4.9–14.3
2	10 (6.1)	3.0–11.0	16 (10.2)	5.9–16.0	7 (4.4)	1.8–8.9
3	2 (1.2)	0.1–4.4	8 (5.1)	2.2–9.8	1 (0.6)	0.0–3.5
4	1 (0.6)	0.0–3.4	0 (0.0)	0.0–2.3	1 (0.6)	0.0–3.5

[a] The most severe grade was counted when fever in the same subject occurred at different grades over the three injections. Grade 1, ≥37.5°C; grade 2, ≥38.0°C; grade 3, ≥39.0°C fever continued for less than a day; grade 4, ≥39.0°C fever continued for >2 days.
[b] CI, confidence interval.

against the Beijing-1 strain induced by formulations L or M was higher than that by the MB-JEV.

Table 6 shows the GMTs after the 3rd injection according to four patterns of injection volume (1st/2nd/3rd injection): 0.25/0.25/0.25 ml, 0.25/0.25/0.5 ml, 0.25/0.5/0.5 ml, and 0.5/0.5/0.5 ml. The number of subjects for whom the dose was changed from 0.25 ml to 0.5 ml was small. Comparing sets of two patterns, such as 0.25/0.25/0.25 ml and 0.5/0.5/0.5 ml of formulations L and M, respectively, there were no statistically significant differences between them. Based on these results, it is considered acceptable to inoculate children <3 years of age with 0.25 ml of any CC-JEV.

Safety in trial 2. In trial 2, vaccine-related adverse events with an incidence of ≥5% are listed in Table 7. In the local reactions, the most common vaccine-related adverse event was injection site erythema. There were no statistically significant differences in the incidences of injection site erythema among group L, group M, and the control group. In the systemic reactions, the most common vaccine-related adverse event was fever. The incidence of fever in group M was statistically higher than that in the control group (confidence interval for group M, 21.2 to 35.7; control group, 9.4 to 20.9). Most of the adverse events in both group L and group M were lower than grade 3 (<39.0°C) (Table 8). No serious vaccine-related adverse event was observed in trial 2. Based on these results, we selected formulation L containing 4 μg of CC-JEV per dose as the optimum dose.

Table 9 shows the vaccine-related adverse events after the 3rd injection according to four patterns of injection volume (1st/2nd/3rd injection): 0.25/0.25/0.25 ml, 0.25/0.25/0.5 ml, 0.25/0.5/0.5 ml, and 0.5/0.5/0.5 ml. The number of subjects for whom the dose was changed from 0.25 ml to 0.5 ml was small. Comparing sets of two patterns, such as 0.25/0.25/0.25 ml and 0.5/0.5/0.5 ml, of formulations L and M, respectively, there were no statistically significant differences between them. Based on these results, it is considered acceptable to inoculate children <3 years of age with 0.25 ml of formulation L.

DISCUSSION

We have developed a new type of JE vaccine, a freeze-dried CC-JEV vaccine derived from Vero cells instead of from mouse brain. The new type of JE vaccine contains a more highly purified antigen than in the previous vaccines, produced by adding an affinity column chromatography step to the manufacturing process used for the production of the MB-JEV. To evaluate the new type of JE vaccine, two series of phase III clinical trials, trials 1 and 2, were conducted as double-blinded randomized parallel-group studies. The trials showed that 4 μg per dose of formulation L has the same immunogenicity as 17 μg per dose of the MB-JEV, and formulation L was renamed Encevac. This is the first report on a direct comparison of immunogenicity and safety in children inoculated with the CC-JEV and MB-JEV derived from the Beijing-1 strain in phase III clinical trials.

After the launch of Encevac in 2011, an additional clinical study using Encevac was recently conducted to investigate its safety and immunogenicity, focusing on the 2nd stage of the Japanese public immunization program in children 9 to 12 years of age (K. Okada, personal communication). The GMT of 21 subjects who had received Encevac at the 1st stage was $10^{2.68}$ when measured prior to the booster injection at the 2nd stage, and the GMT had increased to $10^{3.84}$ by about 1 month after the booster injection of Encevac at the 2nd stage. On the other hand, the GMT of 34 subjects who had received MB-JEV at the 1st stage was $10^{2.37}$ when measured prior to the booster injection at the 2nd stage, and the GMT had increased to $10^{3.65}$ by about 1 month after the booster injection of Encevac at the 2nd stage. No serious vaccine-related adverse events were reported for any of the groups. Thus,

TABLE 9 Adverse vaccine reactions over three injections in four patterns different in vaccine volume in trial 2

Vaccine volumes at 1st/2nd/3rd injections (ml)	Group (injected vaccine type) (*n*)					
	L (formulation L) (163)		M (formulation M) (155)[a]		Control (MB-JEV) (159)	
	No. with adverse reaction/total no. receiving dose	% (95% CI)[b]	No. with adverse reaction/total no. receiving dose	% (95% CI)	No. with adverse reaction/total no. receiving dose	% (95% CI)
0.25/0.25/0.25	14/25	56.0 (34.9–75.6)	11/18	61.1 (35.7–82.7)	8/22	36.4 (17.2–59.3)
0.25/0.25/0.5	9/11	81.8 (48.2–97.7)	6/11	54.5 (23.4–83.3)	5/9	55.6 (21.2–86.3)
0.25/0.5/0.5	0	NA[c]	2/4	50.0 (6.8–93.2)	1/1	100.0 (2.5–100.0)
0.5/0.5/0.5	61/127	48.0 (39.1–57.1)	70/122	57.4 (48.1–66.3)	73/127	57.5 (48.4–66.2)

[a] Two subjects were excluded because they did not receive the 3rd injection.
[b] CI, confidence interval.
[c] NA, not applicable.

this clinical study targeting the booster injection at the 2nd stage has further confirmed the safety and immunogenicity of Encevac.

In the 1880s, incidents of ADEM related to a rabies vaccine that was prepared from rabbit spinal cord were reported, and protein contaminants deriving from the spinal cord were considered to be the cause of ADEM (16, 17). Incidents of ADEM after inoculation with a Japanese encephalitis vaccine were also suspected to be caused by protein contaminants derived from mouse brain. Based on these considerations, attempts have been undertaken to reduce the rate of ADEM by changing the materials of the vaccine from mouse brain to cell culture product. However, the World Health Organization (WHO) finally concluded (18) that no causal relationship exists between ADEM and inoculation with the Japanese encephalitis vaccine derived from mouse brain. Furthermore, the WHO Global Advisory Committee on Vaccine Safety concluded that no evidence exists regarding an increased risk of ADEM associated with administration of the inactivated JE vaccine. In fact, as the ADEM incidence rate of the newly approved CC-JEV in Japan was one case per approximately 1.3 million injections, while the ADEM incidence rate of the MB-JEV was one case per 0.7 to 2.0 million injections, the number of incidents of ADEM has not reduced after the introduction of the CC-JEV in Japan. However, the CC-JEV has the advantages of being able to reduce a possible risk caused by the contamination of unknown adventitious agents derived from mouse brain and to save animals necessary for manufacturing the JE vaccine.

Although the CC-JEVs used in Europe and the United States contain an aluminum hydroxide adjuvant (5), Encevac shows high immunogenicity even at a low vaccine antigen dose, without any adjuvant. Furthermore, Encevac has a 3-year shelf life under refrigerated conditions without containing any preservative, such as thimerosal. As JE is still the most common kind of viral encephalitis in Asia, especially in tropical and subtropical countries, the JE vaccine Encevac is expected to contribute to the prevention of JE.

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Note

Changes in nasopharyngeal carriage of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* among healthy children attending a day-care centre before and after official financial support for the 7-valent pneumococcal conjugate vaccine and *H. influenzae* type b vaccine in Japan

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ABSTRACT

The 7-valent pneumococcal conjugate vaccine (PCV7) and *Haemophilus influenzae* type b (Hib) vaccine reduce nasopharyngeal carriage of vaccine-type bacteria, which may in turn influence the presence of other nasopharyngeal bacterial pathogens. To investigate this possibility, nasopharyngeal carriage of potential pathogens was examined before and after official financial support was provided to offer the PCV7 and Hib vaccines in healthy children attending a day care centre in Japan during 2011–2012. Despite a virtual disappearance of PCV7 serotypes over time, the overall pneumococcal carriage rate remained unchanged. Although others have reported an increase in PCV13 serotypes following PCV7 vaccination, only non-PCV13 serotypes were observed to have increased in this study. The majority of *H. influenzae* isolates were non-typeable and Hib was not found. Our data identified an unexpected pattern of pneumococcal serotype replacement following PCV7. Continuous monitoring of pneumococcal carriage is important for decisions regarding the future of national vaccination policy in Japan.

The 7-valent pneumococcal conjugate vaccine (PCV7) and *Haemophilus influenzae* type b (Hib) vaccine prevent nasopharyngeal acquisition and transmission of 7 serotypes of pneumococci and Hib in children, respectively [1–3]. Vaccinating children with both PCV7 and Hib vaccines offers effective protection against invasive disease due to PCV7 serotypes and Hib in all age groups [3–5]. However, in many countries, the nasopharyngeal flora of PCV7-vaccinated children is immediately occupied by non-PCV7 but PCV13 serotype pneumococci either due to true replacement, unmasking, or capsular switch, resulting in a similar overall pneumococcal carriage rate [1]. As a result, PCV13 vaccination is now a prevailing strategy to prevent against severe pneumococcal disease, including invasive pneumococcal disease (IPD), in the US and Europe. In Korean children, *Streptococcus pneumoniae* serotype 19A is increasingly recognized as a cause of IPD prior to the introduction of PCV7 [6]. In Japanese children, rates of invasive pneumococcal disease (IPD) due to 19A and non-PCV13 serotypes increased soon after the introduction of PCV7 [7].

In Japan, the PCV7 and Hib vaccines were not approved by the Japanese Ministry of Health, Labor and Welfare until January of 2007 and 2008, respectively. Therefore, it was not possible to have children voluntarily vaccinated against Hib and PCV7 until late 2008 and 2009, respectively. In November 2010, the Japanese Ministry of Labour Health and Welfare established a Provisional Special Fund and recommended vaccination of children with Hib vaccine and PCV7 for the Urgent Promotion of Vaccination. After

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this, Hib vaccine and PCV7 were formally added to the immunization schedule for Japanese infants in 2013.

Since February 2011, Hib and PCV7 vaccines have been publically funded for children under 5 years old in Chiba Prefecture. However, because these vaccines were not yet widely accessible in Japan, vaccination rates among infants and younger children at risk were estimated to be 40–60% at the end of 2011. Therefore, the aim of this study was to investigate the nasopharyngeal carriage of bacteria in healthy children before and immediately after official financial support was provided for these vaccinations.

After obtaining written informed consent from at least one parent, a nasal swab was taken from the participating child and the parent was asked to complete a standardized short questionnaire. The study population consisted of 57 children.

Approval for this study was obtained from the Medical Ethical Committee of Chiba University (no.1120).

Children attending a day care centre at Chiba University Hospital were studied from February 2011 to October 2012 with nasopharyngeal swabs taken every 6 months. At least one of their parents worked at Chiba University Hospital. Parents of the participating children completed a brief survey about their PCV7 and Hib vaccination history. Samples of nasopharyngeal flora were obtained from the children with a nylon flocked flexible sterile Copan E-swab according to World Health Organization standard procedures [8]. After sampling, all swabs were directly inoculated in a liquid medium and plated within 1 h at the Microbiology Laboratory of Chiba University Hospital. All swabs were processed by the same laboratory and cultured to detect the presence of *S. pneumoniae*, *H. influenzae* and *Moraxella catarrhalis* according to standard bacteriological procedures for conventional cultures. One pneumococcal colony per plate was subcultured and serotyped by Quellung reaction using type-specific antisera from the Statens Serum Institute (Copenhagen, Denmark). To determine the sequence type (ST) of the isolates, multi-locus sequence typing (MLST) was performed as described previously [9]. STs were determined by an internet database search at http://spneumoniae.mlst.net/.

One *H. influenzae* colony per plate was subcultured and serotyped using a slide agglutination test using six monovalent antisera (serotypes a-f) manufactured by Remel (Remel Inc., Lenexa, KS, USA). Specimens were also inoculated on Hib antiserum agar prepared with Levinthal base and Hib antiserum as described previously [10].

SPSS statistics 18.0 software was used to examine differences in distribution between the studied populations. The crude odds ratio (OR) and Mantel–Haenszel OR stratified by age with 95% confidence intervals (CIs) were calculated using the χ^2 test. A P value of <0.05 was considered statistically significant.

Table 1 shows the baseline characteristics of the children who participated in the study. A total of 57 children aged from 5 months to 6 years were enrolled in the study. Twenty children participated once and 37 children participated more than once with 11, 22 and 4 children participating 2, 3 and 4 times, respectively. During the course of the study, no participants hospitalized with IPD or invasive Hib disease.

The number of non-immunized children and children vaccinated on a catch-up schedule gradually decreased, while the number of fully immunized children increased during this study.

S. pneumoniae, *H. influenzae*, *M. catarrhalis* and *Staphylococcus aureus* were isolated from nasopharyngeal culture as pathogenic bacterial species of interest. Because *S. aureus* was detected at a very low rate ($n = 6$), specific bacterial species refers to *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in this report. The distribution of carriage of each pathogen is shown in Table 2. Overall carriage rates of pathogenic bacteria were 47.6% ($n = 59$) for *S. pneumoniae*, 35.5% ($n = 44$) for *H. influenzae* and 58.1% ($n = 72$) for *M. catarrhalis*. No significant association was found between gender and colonization by specific bacterial species. The age-specific recovery of specific nasopharyngeal pathogens is shown in Fig. 1. *S. pneumoniae* and *M. catarrhalis* carriage rates were observed to decline with age, while *H. influenzae* carriage rates remained almost the same. Younger age (<24 months) was significantly associated with *S. pneumoniae* colonization (OR = 1.639, 95% CI 1.147–2.343, $P = 0.008$). Carriage of *H. influenzae* was not associated with age. *M. catarrhalis* declined with age and was significantly more prevalent among children

Table 1
Characteristics of the children participating in this study.

	Mar. 2011	Oct. 2011	Mar. 2012	Oct. 2012
Children	29	35	32	28
Male	18	20	22	16
Female	11	15	10	12
Age group				
<1 yr	2	4	3	0
1 yr	6	12	10	9
2 yr	9	8	8	9
3 yr	5	5	3	5
4 yr	2	2	4	4
>5 yr	5	4	4	1
PCV7 status				
Fully immunized (4 doses)	4	4	8	13
Catch up[a] (1–3 doses)	11	14	12	9
On going[b] (1–3 doses)	5	12	9	4
Not immunized[c]	9	5	3	2
Hib vaccine status				
Fully immunized (4 doses)	5	5	5	12
Catch up[a] (1–3 doses)	12	10	13	9
Ongoing[b] (1–3 doses)	8	16	12	7
Not immunized[c]	4	4	2	0

[a] Catch up: a child first vaccination started after 7 months old and finished with reduced doses.
[b] Ongoing: a child who has not completed his or her vaccination schedule.
[c] Not immunized: a child who has not been immunized.

Table 2
Characteristics of bacterial isolates.

	Mar. 2011	Oct. 2011	Mar. 2012	Oct. 2012	MLST (No. of isolates)
Total No. of *S. pneumoniae*	**16**	**15**	**14**	**14**	
PCV7 serotypes	7 (43.8%)	3 (20.0%)	1 (7.1%)	0 (0%)	
6B	4	2	0	0	ST902 (5) ST5233 (1)
19F	3	0	1	0	ST8454 (1) ST236 (3)
23F	0	1	0	0	ST242 (1)
Non-PCV13 serotypes	9 (56.2%)	12 (80.0%)	13 (92.9%)	14 (100%)	
23A	2	1	0	0	ST338 (3)
15A	1	2	1	2	ST63 (6)
15C	1	0	1	2	ST199 (4)
34	3	7	1	0	ST7388 (11)
35B	1	2	7	2	ST2755 (12)
37	1	0	0	0	ST447 (1)
15B	0	0	3	0	ST199 (3)
6C	0	0	0	3	ST2942 (1) ST5823 (2)
11A/E	0	0	0	3	ST8737 (3)
10A	0	0	0	1	ST5236 (1)
Non-typeable	0	0	0	1	ST4845 (1)
Total No. of *H. influenzae*	**5**	**4**	**24**	**11**	
Type d	0	0	0	1	
Type e	1	1	4	0	
Type f	1	0	0	0	
Nontypeable Hi	3	3	20	10	
Total No. of *M. catarrhalis*	**19**	**12**	**27**	**14**	

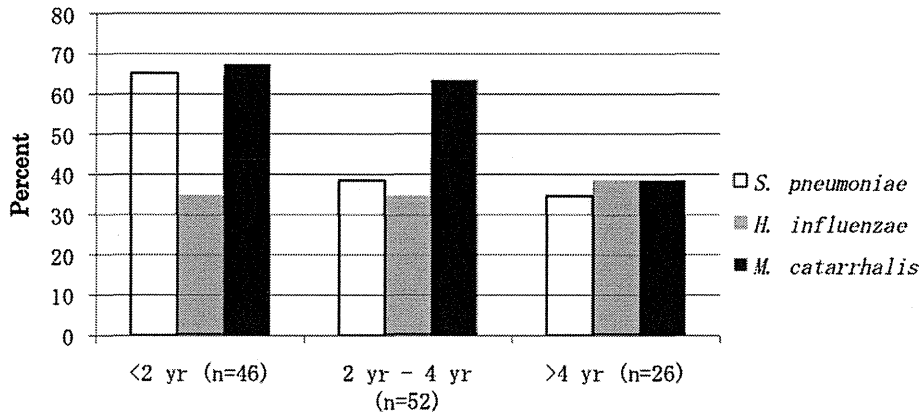

Fig. 1. Recovery of specific nasopharyngeal pathogens with age.

under 48 months (OR = 1.857, 95% CI 1.073–3.214 $P = 0.006$). Almost one third ($n = 45$, 36.3%) of the cases had only one respiratory pathogen. More than one pathogen was colonized in 56 cases (45.2%). Thirty-six cases (29.0%) had two respiratory pathogens and 20 children (16.1%) had three species. Even when the influence of age was eliminated using the Mantel–Haenszel test, a positive association was noted for co-colonization with *S. pneumoniae* and *M. catarrhalis* (OR 4.878, 95% CI 1.442–16.495, $P = 0.009$). No significant associations were observed between the presence of *H. influenzae* and colonization with the other two bacterial species.

We then analyzed the characteristics of 59 *S. pneumoniae* and 44 *H. influenzae* isolates. Near complete eradication of PCV7 serotype carriage was observed within 2 years of announcement of the Provisional Special Fund recommendation for PCV7 immunization. The 6B and 19F PCV7 serotypes were also effectively eliminated following vaccination (Table 2). Although previous studies have reported vaccination to produce an emergence of PCV13 serotypes 6A and 19A, only non-PCV13 serotypes were identified in this study. In PCV7 immunized children (including on going immunization schedule), 50 *S. pneumoniae* strains were isolated, whereas 9 *S. pneumoniae* strains were isolated from PCV7 non-immunized children. Forty-two non-PCV13-type strains and 8 PCV7-type strains were isolated from PCV7 immunized children. Six non-PCV13-type strains and 3 PCV7-type strains were isolated from unvaccinated participants. There was no significant association between the *S. pneumoniae* serotypes and PCV7 immunization status. There were 4 children who participated in this study 3 or 4 times, and carried a PCV7-type strain at the first culture. The PCV7 immunization status of all 4 children did not change during this study. Among them, one child acquired a non-PCV13-type strain and three did not carry any *S. pneumoniae* strains in the last culture. Furthermore, we performed MLST analysis and identified the sequence type (ST) of each serotype (Table 2). Most of isolates with the same serotype had one sequence type (ST).

Next, the capsular serotypes of 42 *H. influenzae* isolates were analyzed and 8 out of 42 (19%) of them were found to be capsulated, after which they were categorized as type d, e, or f (Table 2). No colony was identified on Hib antiserum agar.

Since bacterial colonization depends on numerous factors, including economic and environmental variables as well as host-related factors and vaccination effects, bacterial carriage rates vary widely among different studies and geographical sites [11]. The objective of the present study was to establish the prevalence and specific features of *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* strains circulating amongst day care centre attendees in Japan.

In our study, a majority of children (81.5%) harbored at least one potential respiratory pathogen. Rates of Hib and PCV7 vaccination were high among subjects even before public funding became available. This might be attributable to greater interest among parents regarding their children's health, since at least one parent of each child was working at Chiba University Hospital.

This study shows that the risk of being colonized by *S. pneumoniae* is not associated with colonization by *H. influenzae* but positively associated with colonization by *M. catarrhalis*. The risk of being colonized by *H. influenzae* was not associated with colonization by *M. catarrhalis*, which is consistent with the findings of a previous report [12].

After the introduction of PCV7 vaccination, the prevalence of PCV13 serotypes 6A and 19A has been reported to increase, while PCV7 serotypes are known to become less dominant. PCV13, including serotypes 6A and 19A, replaced PCV7 in vaccination schedules in the US in 2010. Presently, PCV7 is being gradually replaced with PCV13 worldwide. In addition, an increase in non-PCV13 serotypes 15A and 22F has been reported in the US [13]. In our study, carriage of vaccine type strains decreased significantly after PCV7 vaccination became publically funded. Unlike the findings reported in the US and elsewhere, an increase in non-PCV13 serotypes, including serotypes 6C, 15A, 15C, 35B and 11A/E, was observed as opposed to PCV13 serotypes. A similar prevalence of pathogens has been reported in Japanese pediatric patients with IPD [7]. Spread of microorganisms is commonplace in the era of globalization. As such, replacement of the PCV7 vaccine with a PCV13 vaccine may have little efficacy, even in those areas that are currently observing emergence of PCV13 serotypes in the setting of PCV7 vaccination. Prevention and control of pneumococcal infections in young children will require the development of new vaccination strategies aimed at targeting additional serotypes or other antigens.

Introduction of Hib vaccination led to a significant reduction of Hib disease and carriage in both vaccinated and unvaccinated children due to a herd immune effect [14]. More than 80% of children in our study were vaccinated against Hib, and no Hib strain was recovered in any child. Kuroki et al. reported a Hib carriage rate of 0.84% among healthy Japanese children prior to introduction of the Hib vaccine [10]. Specific data regarding the prevalence of Hib carriage prior to introduction of the Hib vaccination are not available in this setting and the absence of Hib isolates is likely to be the result of a very low prevalence of Hib carriage alone. *H. influenzae* capsular type d, e and f were present in small amounts but detected every time. Invasive disease due to *H. influenzae* type d, e or f is rare, but needs to be considered as a possibility. Although a randomized controlled study reported that no changes in carriage rate with *H. influenzae* and *M. catarrhalis* were observed after vaccination with 2 or 3 of doses PCV7 [15], the carriage rate of *H. influenzae* and

M. catarrhalis in this study seems to have increased after official financial support for the PCV7 and Hib vaccine was introduced. Higher PCV pressure following a 4 doses schedule and nationwide introduction in the routine infant vaccination schedule may induce bacterial shifts. We should monitor the colonization status of immunized children to evaluate this potential phenomenon.

Continuous surveillance of carriage of invasive disease pathogens will allow us to establish the effect of conjugate vaccine use on *S. pneumoniae* and *H. influenzae* serotype distribution.

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Conflict of interest

None.

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Original article

IgG levels against 13-valent pneumococcal conjugate vaccine serotypes in non pneumococcal conjugate vaccine immunized healthy Japanese and intravenous immunoglobulin preparations

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ABSTRACT

No studies showed specific antibody levels against all serotypes covered by 13-valent pneumococcal conjugate vaccine (PCV13) among polyclonal intravenous immunoglobulin (IVIG) products. Our study aimed to assess whether we could expect the efficacy of IVIG therapy for invasive pneumococcal disease (IPD) and to clarify the age group which should be recommended for IVIG therapy in case of IPD. Serotype-specific immunoglobulin G (IgG) levels against PCV13 serotypes were measured in four IVIGs which were produced from Japanese donors who were not immunized with any pneumococcal conjugate vaccines (PCVs), and in the serum of 160 non-PCV immunized Japanese subjects, by enzyme-linked immunosorbent assay. The functional opsonic activities of the IVIGs against serotypes 6B and 19A were assessed by a multiplexed opsonophagocytic killing assay. Japanese infants aged <2 years had a geometric mean IgG concentration of <0.35 μg/ml against several serotypes. Serotype-specific IgG concentrations varied among IVIGs. In general, IgG antibodies against serotypes 6A, 14 and 19A were higher in each IVIG. Although opsonization indices also varied among preparations, each IVIG had the ability to opsonize both serotypes 6B and 19A. This study suggests that routine immunization with PCV is important for prevention of IPD, especially for children <2 years old and IVIGs might be effective for IPD patients.

1. Introduction

Streptococcus pneumoniae, a leading cause of pneumonia, bacteremia, and meningitis, causes substantial morbidity and mortality. Worldwide, *S. pneumoniae* causes an estimated 1.6 million deaths annually, of which about 830,000 occurred among children <5 years of age in the year 2000 [1]. Invasive pneumococcal disease (IPD) sometimes causes fatality and sequelae, especially for people with underlying disease [2].

El-Nawawy et al. reported a randomized controlled study of polyclonal intravenous immunoglobulin (IVIG) in pediatric sepsis syndrome, where patients showed a significant reduction in mortality [3]. IVIG administration might be an effective therapy against IPD. However, little has been reported about IVIG treatment for IPD, except reports about prevention of infections in common variable immune deficiency [4] and patients without a spleen [5].

Food and Drug Administration (FDA) regulations, which also apply to IVIG, require that all immunoglobulin product lots possess a minimum level of antibodies to measles, diphtheria, and poliomyelitis (which are provided in FDA regulation 21 CFR 640.104), but not serotype-specific antibodies to *S. pneumoniae*. In 2000, the World Health Organization (WHO) described guidelines for the pneumococcal enzyme-linked immunosorbent assay (ELISA) as an international standard protocol to quantify the concentrations of antibody levels against pneumococcal serotype-specific polysaccharides (http://www.vaccine.uab.edu). To measure pneumococcal serotype-specific antibody levels against all 13-valent

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pneumococcal conjugate vaccine (PCV13) serotypes by this ELISA would be time-consuming and expensive. A few studies showed specific antibody levels against several selected pneumococcal serotypes among IVIG products [6], and there is no report about serotype-specific antibody levels against all serotypes covered by PCV13, serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F.

IPDs caused by serotypes (especially 19A) not covered by the 7-valent pneumococcal conjugate vaccine (PCV7) increased after the introduction of PCV7 [7]. In Japan, as PCV7 was introduced in 2010, IPDs by non-PCV7 serotypes would be forecast to increase in the future. Adjuvant therapy with antibiotics might be needed for patients with IPDs caused by non-vaccine serotypes. Therefore, it is important to investigate specific antibody concentrations against non-PCV7 pneumococcal serotypes in IVIG products.

In this study, we measured serotype-specific immunoglobulin G (IgG) antibody levels against all pneumococcal serotypes covered by PCV13 (PCV7 serotypes + 1, 3, 5, 6A, 7F and 19A) in commercially available IVIG preparations, to assess whether we could expect the efficacy of IVIG therapy for patients with IPD, and in the general Japanese population, to clarify which age group should be recommended IVIG therapy in case of IPD, using WHO ELISA protocol. In addition, we assessed functional opsonization of serotype 6B and 19A by IVIG preparations using a multiplexed opsonophagocytic killing assay (MOPA), because serotype 6B frequently causes IPD in Japan [2] and IPD caused by serotype 19A is an increasing problem at present.

2. Materials and methods

2.1. Serum samples

The serum samples were collected between 2006 and 2012 from healthy Japanese children and young adults who were not immunized pneumococcal conjugate vaccines (PCVs). Of these samples, 146 samples were collected before the introduction of PCV7 and 14 samples were collected after the introduction of PCV7. The number of samples analyzed per group aged <2 years old, 2–4 years old, 5–9 years old and young adults (18–23 years old) were 38, 21, 51and 50 with respectively. Vaccination with the 23-valent pneumococcal polysaccharide vaccine (PPV23) is only recommended in Japan for risk groups, such as children over 2 years old with high risk underlying diseases for IPD and elderly over 65 years old. PPV23 is not used as a routine vaccination. Although no information about vaccination with PPV23 was available for participants in this study, usage can considered to be rare. Serum samples were stored at <−20 °C until analysis. The study was conducted to comply with the ethical standards of Chiba University on human experimentation, and with the Helsinki Declaration. The informed consent was obtained from subjects or their guardians.

2.2. IVIG preparations

Five lots each of four commercial IVIG preparations (identified here as A to D), were analyzed in this study. Each IVIG product was characterized by different treatments during production, as follows: product A was treated with polyethylene glycol; product B was a freeze-dried preparation treated with polyethylene glycol; product C was an acidic preparation subjected to incubation at pH 4; and product D was a freeze-dried, sulfonated preparation. The IVIG preparations were produced from the plasma of voluntary Japanese blood donors between 2008 and 2011. In Japan, PCV7 was introduced for children under 10 years old in 2010. Blood donors must be at least 16 years old in Japan. Therefore all preparations were produced from the plasma of donors who were not immunized with PCVs. All IVIGs were provided as 50 mg/ml solutions. Reconstituted IVIGs were diluted 1:200 with PBS for the ELISA. When the results were assessed, specific pneumococcal IgG levels in IVIG preparations were re-calculated the experimental data by two hundred times.

2.3. Pneumococcal serotype-specific IgG assay

Pneumococcal serotype-specific IgG was measured with the WHO approved ELISA using the standard reference serum (007sp), and C-polysaccharide and 22F polysaccharide absorptions (http://www.vaccine.uab.edu) as previous reported [8]. Optical density data were converted to antibody concentrations with a computer program which used a four parameter logistic-log method to perform a curve-fitting procedure. Details of the procedure are provided at http://www.vaccine.uab.edu.

We assessed the concentrations of antibodies against all serotypes covered by PCV13, serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F. The WHO suggested a serotype-specific IgG concentration of 0.35 μg/ml as a putative measure of protection at a population level against IPD in infants after immunization with PCV [9]. This reference antibody concentration of ≥0.35 μg/ml has been determined through a pooled analysis of data from the PCV efficacy trials with invasive disease end-points that have been completed to date. This threshold does not necessarily predict protection by naturally acquired immunity in an individual subject. Additionally, the relevance of this threshold to passively acquired immunity by using IVIG has not been established.

We used serotype-specific IgG antibody >0.35 μg/ml as the temporary threshold in this study.

2.4. Multiplexed opsonophagocytic killing assay (MOPA)

In order to measure the functional activity of the anti-pneuomococcal antibodies in the IVIG products, a multiplexed opsonophagocytic killing assay (MOPA) was performed. Opsonization indices (OIs) were measured for serotypes 6B and 19A based on the phagocytosis of antibiotic-resistant target bacteria, as previously described [10]. For total IVIG, the total IgG concentration at the first dilution of the IVIG (1:4) was 12.5 mg/ml. Pneumococcal strains, of serotypes 6B and 19A, resistant to spectinomycin or trimethoprim, respectively, were obtained from BEI Resources (www.beiresources.org). Differentiated HL-60cells (ATCC, Manassas, VA) were allowed to phagocytose *S. pneumoniae* in the presence of IVIG antibodies and baby rabbit complement (Pel-freez Biologicals, Rogers, AR). 10 μl aliquots of the reaction mixture were plated onto two different Todd-Hewitt-0.5% Yeast extract agar plates containing appropriate antibiotics. The bacterial colonies were counted after overnight incubation at 37 °C in an atmosphere containing 5% CO_2. The OI was defined as the greatest dilution of IVIG that killed 50% of bacteria. The OIs were determined using opsotiter3 software according to the WHO protocol (www.vaccine.uab.edu/UAB-MOPA).

2.5. Statistical analysis

Geometric mean IgG concentrations (GMCs) with 95% confidence intervals (CI) were calculated for each age group. The average for the five IVIG lots was shown using bar graphs with error bars representing one standard deviation. All statistical analyses were performed using GraphPad Prism v5 software (GraphPad Software, Inc.). Calculations of *p*-values were performed with one-way ANOVA, followed by Tukey's multiple comparison test, to compare the individual serotype-specific IgG levels for all 13 serotypes in each age group, or each IVIG preparation. Calculations of *p*-values were performed with two-way ANOVA followed by the

Bonferroni post-hoc test, to compare the serotype-specific IgG levels against individual serotypes among IVIG preparations.

3. Results

3.1. Seroprevalence of pneumococcal serotype-specific IgG antibodies in the Japanese population

Fig. 1 illustrates GMCs of the IgG levels for each of the 13 serotypes stratified by age group. Overall, infants aged <2 years had low GMCs. Infants aged <2 years had GMCs <0.35 μg/ml against serotypes 1, 4, 7F, 9V, 18C and 23F. GMCs against all serotypes increased with increasing age. The GMC of IgG levels against serotype 14 was significantly higher than those against serotypes 1, 4, 3 and 7F in each age group ($p < 0.05$), except in the 2–4 years old group.

3.2. Pneumococcal serotype specific IgG antibody levels in IVIG preparations

Specific IgG levels against all serotypes included in PCV13 were measured by the ELISA protocol in 5 lots of each different IVIG preparation. The results are summarized in Fig. 2. All IVIG preparations contained relatively abundant IgG antibodies against all serotypes included in PCV13. There was no significant variation in IgG levels against all serotypes between individual lots of IVIG. However, there were significant differences in IgG levels to individual serotypes in each IVIG. IgG levels against serotypes 6A, 14 and 19A were significantly higher than those against serotypes 4, 7F and 9V in each IVIG preparation ($p < 0.05$). Comparing IgG levels to each serotype among the IVIG preparations, product B had consistently lower IgG levels than other products, with significantly lower levels of IgG against serotypes 3, 5, 6A, 14 and 19A than the other three products ($p < 0.05$).

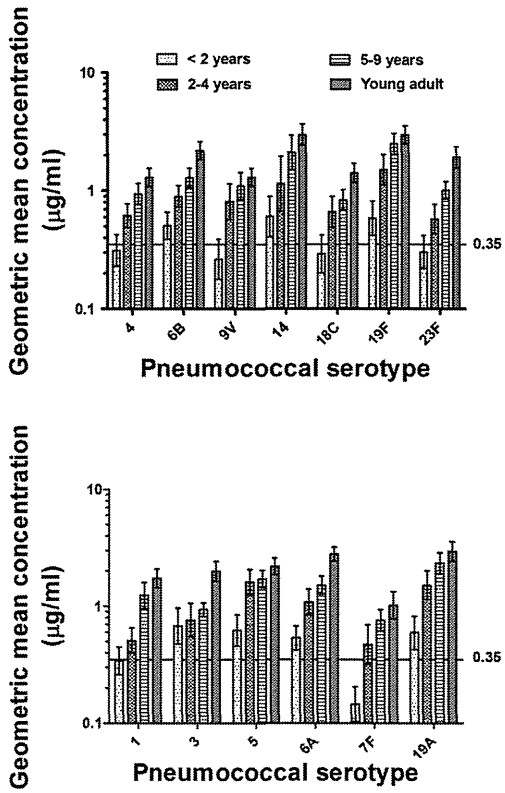

Fig. 1. Age-stratified geometric mean IgG concentrations (GMCs) with 95% confidence interval (CI). In the upper panel the PCV7 serotypes are presented and in the lower panel the other six serotypes are presented. The straight lines indicate the 0.35 μg/ml threshold concentration.

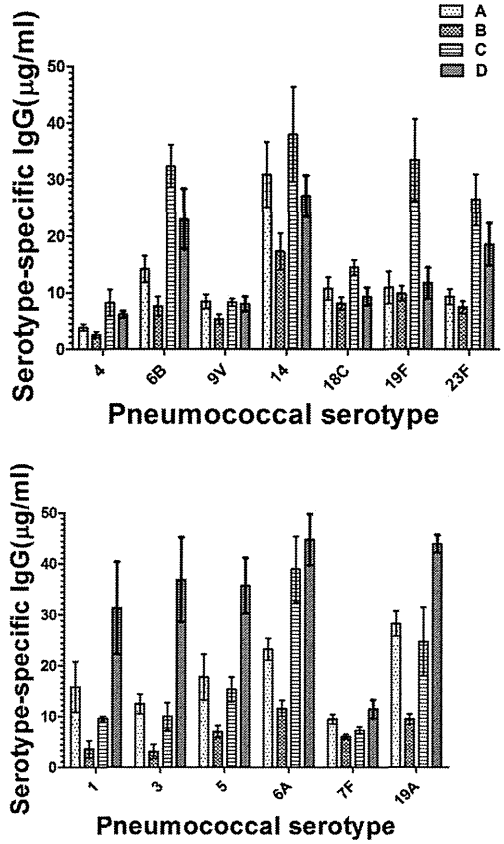

Fig. 2. The average serotype-specific IgG levels of five IVIG lots shown by bar graph with error bar of standard deviations. In the upper panel the PCV7 serotypes are presented and in the lower panel the other six serotypes are presented.

3.3. Opsonization index (OI)

MOPA for serotypes 6B and 19A was performed for 3 lots of each IVIG preparation. Table 1 shows the mean OIs of the three products. In general, each IVIG preparation had the ability to opsonize both serotypes 6B and 19A. Although product A did not have the highest levels of IgG against serotypes 6B and 19A when compared with the other three products, it had the highest OIs for both serotypes 6B and 19A. Product B had the lowest levels of IgG against serotype 19A of the four products; however, it had a higher OI than product C. The correlation coefficients couldn't be calculated because there

Table 1
Opsonophagocytic killing assay titers of polyclonal intravenous immunoglobulin preparations.

Product	Mean OI of 3 lots of each product	
	6B	19A
A	38058	1906
B	512	557
C	1305	254
D	5268	1123

The Ig concentration in the first dilution (1:4) was 12.5 mg/ml.
Capacity represents OI—maximum dilution of polyclonal intravenous immunoglobulin preparation at which 50% of the pneumococcal CFU were killed.
OI; opsonization index, CFU; colony-forming unit.

were only few sample data and their data didn't follow normal distribution.

4. Discussion

We have presented here data showing the levels of specific IgG against all serotypes included in PCV13, both in four IVIG preparations and in a healthy Japanese population. To our knowledge, this is the first report to have reported the levels of IgG against all PCV13 serotypes in both IVIG preparations and the Japanese population, using the WHO protocol.

In this study, infants aged <2 years had GMCs of <0.35 μg/ml against serotypes 1, 4, 7F, 9V, 18C and 23F and children ≥2 years old had GMCs of >0.35 μg/ml against all serotypes included in PCV13, even though they were non PCV7 immunized population. The age-stratified dynamics of the IgG concentrations were rather different to those reported in results from a similar study in the Netherlands [11]. We could not easily compare our results to theirs, because there were differences in sample numbers and they measured serotype-specific IgG concentrations by using a multiplex bead-based assay. Therefore, we compared the trend of our data to their data. Although they demonstrated that both infants aged <2 years and children aged 2–4 years had GMCs <0.35 μg/ml against most serotypes, our data showed that children aged 2–4 years had GMCs higher than the threshold. However, the incidence of IPD among infants aged <2 years was higher than that seen among other groups of children, both in the Netherlands [12] and Japan [13]. It is unclear why the incidence of IPD in Dutch children aged 2–4 years age group is low compared in Dutch <2 years, despite lower GMCs in this age group. Our results might suggest a possible correlation between the incidence of IPD and serotype-specific IgG levels when stratified by age group. The differences of serum GMCs between Dutch and Japanese might be caused by the differences of immune responses for *S. pneumoniae* in race and the degree of pneumococcal exposure in the regions. So far, there is no comparable data on the natural immune response of IPD between Dutch and Japanese population. However, according to the immunological data of recent clinical trials of PCV13 in two countries, the IgG levels after the immunization of PCV13 among both Japanese population and Dutch population increased more sufficiently than the IgG levels before the immunization of PCV13 [14, 15]. Therefore both Japanese and Dutch population is suggested to have good immunological responses for PCV13.

The development of natural immunity to pneumococci is likely due to exposure to carriers and/or to exposure to pneumococcal mucosal diseases such as otitis media, bronchitis or sinusitis. Other bacterial species may also induce cross-reacting antibodies to certain serotypes[16, 17]. Masuda et al. reported that all the children younger than 1 year carried *S. pneumoniae* and the carriage rate decreased with age in Japanese day-care centers [18]. We suggested that frequent exposure of pneumococci might cause to higher GMCs in Japanese children ≥2 years old. Most IPD pediatric patients are under 2 years old prior to introduction of PCVs.

We consider low pneumococcal serotype-specific IgG level is one risk factor of IPD. The routine immunization of infants prior to the exposure of pneumococci, exactly immunization from 2 month-old infants, with PCV may be effective for the prevention of IPD. In Japan, PCV7 was introduced as the voluntary immunization program from 2010. Japanese infant could be vaccinated PCV7 from April 2013 and finally can be vaccinated PCV13 from November 2013 as national immunization program. Therefore, it is suggested that most Japanese might have serotype-specific IgG titer against PCV13 serotypes (except PCV7 serotypes) which is about the same level as the results in this study.

Our results for the IgG levels in the IVIG preparations contrast with a similar study by Mikolajczyk et al. [6]. These authors showed antibody levels to serotypes 14 and 19F are normally several-fold higher than those to types 4, 6B and 9V. In our study, all of the IVIG preparations contained relatively high levels of IgG against serotype 6A, 14 and 19A. The difference might be explained by the variations in the age of donors, as well as differences in both the frequency of occurrence and the epidemiology of *S. pneumoniae* in these different countries.

Polyclonal IVIG has been shown to aid phagocytosis by coating bacteria with IgG and activating the complement cascade, or by producing opsonins by activation of the 'alternative complement pathway' [19]. Thus, specific antibody against *S. pneumoniae* may be critical. The minimum concentration of serotype-specific pneumococcal antibody required to be effective against IPD is unclear. We calculated a proposed dosage rate that would be effective against IPD by using the threshold of 0.35 μg/ml of specific antibody in serum. In Japan, the dose for severe infections such as sepsis and meningitis is 100–150 mg/kg IVIG with a concentration of 50 mg/ml as a single dose. If a 10-kg child was administered 150 mg/kg as a single dose, estimating a circulating blood volume of 800 ml, a 30 ml of the IVIG solution will be needed. Although the required serotype-specific IgG concentration against each serotype in an IVIG preparation would be 9.3 μg/ml (0.35 μg/ml × 800 ml divided by 30 ml) to achieve the threshold level in the child's blood, none of the IVIG preparations contain 9.3 μg/ml of serotype-specific IgG antibodies against serotype 4 and 9V. In particular, product B does not have the required IgG concentration against most serotypes, except serotypes 6A, 14, 19A and 19F. In a previous study, mortality has been significantly reduced among children with sepsis syndrome who received polyclonal IVIG at a dose of 400 mg/kg for 3 days [3]. If a 10-kg child is administered 400 mg/kg of IVIG solution which is volume of 80 ml as a one-time dose, serotype-specific IgG concentration in an IVIG preparation would only need to be 3.5 μg/ml (0.35 μg/ml × 800 ml divided by 80 ml) to achieve the threshold level. All IVIG preparations contain serotype-specific IgG against each PCV13 serotype at levels >3.5 μg/ml. It is suggested that patients with IPD, especially aged <2 years, might require sufficient dose of IVIG therapy because they have low serotype-specific IgG against most PCV13 serotypes. Therefore, we propose IVIG preparations would be more effective for patients with IPD if used at higher doses than the regular dose (100–150 mg/kg).

S. pneumoniae has other common structural components, such as Pneumococcal cell wall polysaccharide (C-polysaccharide), pneumococcal surface protein A (PspA), pneumococcal surface adhesion A (PsaA) and pneumolysin. Musher et al. measured mean IgG reactive against C-polysaccharide by ELISA in 15 healthy young adults and in 126 randomly selected hospital patients of all ages [20]. In three groups of patients (3 with acute purulent tracheobronchitis, 13 with non-bacteremic pneumococcal pneumonia, and 14 with *S. pneumoniae* bacteremia) at the time of admission, mean antibody levels were higher than the IgG levels in healthy adults. In the results, they suggested that naturally present anti-C-polysaccharide IgG did not protect against the evolution of acute pneumococcal infection from colonization to acute purulent bronchitis, from bronchitis to pneumonia, or from pneumonia to bacteremia. Holmlund et al. measured the antibody concentrations against PspA, PsaA and Ply by enzyme immunoassay in serum samples of 51 Filipino pregnant women, in six consecutive serum samples of 173 infants (samples from 7 to 48 weeks of age) and collected nasopharyngeal swabs from the infants [21]. The GMC of anti-PspA and -Ply decreased until 18 weeks of age and started to increase thereafter. The GMC of anti-PsaA in the infants increased significantly by age and reached the GMC of the mothers already at 14 weeks of age. High maternal anti-Ply antibodies were negatively

associated with the risk of pneumococcal carriage. According to these studies, IVIG preparation might contain specific IgG against these components, but, to our knowledge, there are no data about it. IVIG preparations will be suggested to have more effects for IPD patients, if specific IgG levels against their components in IVIGs would be measured.

There was no certain trend in our study between the pneumococcal ELISA and OI results, and similar results have been reported from another study [6]. The lack of correlation may be attributable to differences in antibody avidity or complement fixation activity, which were not examined in this study. In addition, the OI can have high intra-and/or inter-assay variability. While a functional test such as the OI is desirable, more research needs to be performed for assay optimization. Although there was the lack of correlation in the present study, these four IVIG preparations do have opsonophagocytic capacity against serotypes 6B and 19A. Although it might be necessary to measure OIs for other serotypes including PCV13, we couldn't measure them simultaneously because materials and labwares for MOPA were limited. Serotype 6B frequently causes IPD in Japan and IPD caused by serotype 19A is an increasing problem at present. Therefore we considered OIs of these serotypes were useful information. Further studies would be expected to evaluate opsonophagocytic activities of IVIG preparations.

In conclusion, we measured the IgG levels against PCV13 pneumococcal serotypes in both commercial IVIG preparations and the healthy non-PCV7 immunized Japanese population. The use of IVIG might be an adjuvant therapy for patients with IPD, especially for patients <2 years old. The results of our experiments support the concept that effective IgG levels for the treatment of IPD caused by all PCV13 serotypes would be achieved in human subjects after infusion of IVIG at an increased dose than the regular dosage used in Japan.

Conflict of interest

None.

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The incidence of pediatric invasive *Haemophilus influenzae* and pneumococcal disease in Chiba prefecture, Japan before and after the introduction of conjugate vaccines

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ABSTRACT

The *Haemophilus influenzae* type b (Hib) vaccine and the heptavalent pneumococcal conjugate vaccine (PCV7) were introduced in Japan in 2008 and 2010, respectively. In 2011, immunization with these two vaccines was encouraged throughout Japan through a governmental program. Children treated in Chiba prefecture for culture-proven invasive *H. influenzae* disease (IHiD) and invasive *Streptococcus pneumoniae* disease (IPD) were identified in a prefectural surveillance study from 2008 to 2013. The incidence rate ratio (IRR) and its confidence interval (CI) were calculated to compare the 3 years before and after governmental financial support for vaccination. The average number of IHiD and IPD cases among children <5 years of age in 2011–2013 decreased 84% (IRR: 0.16, 95% CI: 0.09–0.26, $p < 0.0001$) and 51% (IRR: 0.49, 95% CI: 0.37–0.63, $p < 0.0001$) compared with those occurring in 2008–2010. The most common non-PCV7 serotype encountered in 2011 and 2013 was 19A. After governmental subsidization of Hib and PCV7 vaccination, IHiD and IPD decreased in Chiba prefecture, Japan. Continuous surveillance is necessary to determine the effectiveness of these two vaccines and for detection of emerging invasive serotypes.

1. Introduction

Haemophilus influenzae (*H. influenzae*) and *Streptococcus pneumoniae* (*S. pneumoniae*) are major causes of serious invasive infection, resulting in high mortality and morbidity in children due to meningitis, septicemia and pneumonia. Over the past decades, the incidence of serious infections due to strains of *H. influenzae* and *S. pneumoniae* having reduced susceptibility to penicillin and broad-spectrum cephalosporins has been steadily increasing in Japan [1,2]. The emergence of these strains has made the selection of antibiotics for treatment more difficult. The introduction of *H. influenzae* type b (Hib) conjugate vaccine and heptavalent pneumococcal conjugate vaccine (PCV7) has dramatically decreased the incidence of invasive Hib and invasive pneumococcal disease (IPD), respectively, all over the world [3–7]. However, in the United States and some other countries, surveillance studies following the introduction of PCV7 have demonstrated an increased prevalence of IPD caused by non-PCV7 serotypes, such as 6A, 19A, 15A, and 35B, suggesting that non-vaccine serotypes are emerging and replacing the vaccine serotypes [8–11]. These two vaccines have been included in the routine immunization program in Japan since April 2013, and the 13-valent pneumococcal conjugate vaccine (PCV13) was introduced in November 2013.

The targeted age group for both Hib conjugate and pneumococcal conjugate vaccination is children <5 years of age. The standard Japanese vaccination schedule for Hib conjugate vaccine consists of 3 doses, one administered at each of the ages of 2, 3, and 4 months, and then a booster at the age of 12–18 months. The standard Japanese vaccination schedule for PCV7 contains 3 doses, at

Abbreviations: Hib, *Haemophilus influenzae* type b; PCV7, heptavalent pneumococcal conjugate vaccine; IPD, invasive pneumococcal disease; PCV13, 13-valent pneumococcal conjugate vaccine; IHiD, invasive *Haemophilus influenzae* disease; IRR, incidence rate ratio; CI, confidence interval; NTHi, non-typeable *Haemophilus influenzae*; ST, sequence type.

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Table 1
Number of cases of invasive *Haemophilus influenzae* disease, by year.

	2008	2009	2010	2011	2012	2013	Total
Meningitis	23	18	29	7	4	0	81
Pneumonia	4	6	7	3	1	0	21
Bacteremia without focus	5	3	7	2	2	0	19
Cellulitis	2	1	3	0	0	1	7
Arthritis	2	3	1	0	0	0	6
Epiglottitis	0	1	1	1	0	0	3
Others	2	0	0	1	0	1	4
Total	38	32	48	14	7	2	141
Baseline population[a]	267,191	268,011	268,031	268,670	262,986	256,961	
Incidence[b] (95% CI)	13.5(9.4–18.7)	11.2(7.6–16.0)	16.8(12.3–22.5)	4.1(2.0–7.3)	1.9(0.6–4.4)	0.4(0.1–2.8)	

CI, confidence interval.
[a] Population <5 years of age.
[b] Cases/100,000 population <5 years of age.

the ages of 2, 3, and 4 months, and then a booster at the age of 12–15 months. After the Hib conjugate and PCV7 vaccines were included in the routine immunization program, these two vaccines were strongly recommended by the Vaccination Law and were given free of charge to all children <5 years of age in Japan.

Accurate, up-to-date information on the incidence of invasive *H. influenzae* diseases (IHiD) and IPD is fundamental for estimating vaccine efficacy and for formulating country-appropriate decision-making regarding management of Hib and pneumococcal conjugate vaccination. However, there is little information regarding the incidence of IHiD and IPD in children in Japan. The purpose of this study was to clarify the incidence of IHiD and IPD in children in the prefecture of Chiba, Japan, using population-based surveillance before and after the introduction of the Hib and pneumococcal conjugate vaccines.

2. Materials and methods

To determine the precise incidence of IHiD and IPD in Chiba prefecture, we conducted a survey during the 2008–2013 time period. Chiba prefecture is one of 47 prefectures in Japan, and is located in the middle of the country. The population in Chiba prefecture is about 6 million, which represents about 5% of the population of Japan. The population of children less than 5 years of age in Chiba prefecture in 2008, 2009, 2010, 2011, 2012, and 2013 was 267,191, 268,011, 268,031, 268,670, 262,986, and 256,961, respectively. We implemented a reporting system for IHiD and IPD in all 58 hospitals in Chiba prefecture, and 11 hospitals located in the surrounding area, that have pediatric wards, to determine the precise population-based incidence in Chiba prefecture. This active surveillance was conducted from 1 January 2008 through 31 December 2013. If the doctors in the targeted hospital treated children aged <16 years who lived in Chiba prefecture for IHiD or IPD, they used standardized case report forms to record patient and laboratory information, and then sent the case report form to Chiba University Hospital by fax or In addition to the reporting system, a written questionnaire was sent to enrolled hospitals twice a year for the identification of unreported cases. The case report form and the questionnaire included the clinical diagnosis, patient's age, underlying disease, vaccination history, treatment and prognosis. Invasive disease was defined as any disease where *S. pneumoniae* and *H. influenzae* was identified in normally sterile body fluids such as blood, cerebrospinal fluid, bone aspirate, or synovial fluid. All recorded pneumonia, cellulitis, and epiglottitis cases were confirmed by the presence of *S. pnemoniae* or *H. influenzae* in blood culture. If the isolated strains had been stocked in the hospital, the strains were sent to Chiba University where serotypes were analyzed. Serotypes of *H. influenzae* isolates were identified by coagulation using antiserum purchased from Denka Seiken (Tokyo, Japan). Serotypes of pneumococcal isolates were identified by the capsular quelling reaction, using antiserum purchased from the Statens Serum Institut (Copenhagen, Denmark). All collected strains were sent to the National Institute of Infectious Diseases, where serotypes were confirmed using the same methods. All statistical analyses were performed by using SAS software version 9.3 (SAS Institute, Cary, NC, USA) and the *R* statistical program, version 2.13. The total incidence rate for children within the 0–4 year age group was calculated for the period 2008–2013, as the number of those with IHiD or IPD per 100,000 in the Chiba prefecture. To compare disease ratios in 2008–2010 with those in 2011–2013, the incidence rate ratio (IRR) and its 95% confidence interval (CI) were calculated. This study was approved by Chiba University Ethics Committee Number 666. Informed consent for collection and use of patient information and specimens was obtained from each parent/guardian.

3. Results

3.1. IHiD

During the 6 years of this study, 141 patients were diagnosed with IHiD. Among these, 81 had meningitis, 21 had pneumonia, and 19 had bacteremia without a focus (Table 1). The highest annual incidence rate of IHiD and *H. influenzae* meningitis among children <5 years of age during the study period both occurred in 2010, and were 16.8 and 10.4 per 100,000, respectively, whereas the corresponding lowest annual incidence rates both occurred in 2013, and were 0.4 and 0, respectively (Fig. 1). The ages of onset of IHiD were available for all 141 patients: 53 (37.6%) were in the 0 year old subgroup, 44 (31.2%) in the 1 year old subgroup, 31 (22.0%) in the 2–4 year old subgroup, and 13 (9.2%) in the 5 years or older subgroup. Males constituted 50% of the subjects (70/141). At least one underlying condition was documented in 14 (9.9%) of the 141 IHiD patients. These included congenital anomaly/syndrome ($n=5$), low birth weight infant ($n=4$), malignancy ($n=3$) and others ($n=2$). Among IHiD cases at age 5 and over, 61.5% had underlying disease. Of the 141 study patients, 8 patients (5.7%) developed permanent neurological complications; 7 of the 8 patients with neurological sequelae had meningitis and one patient had deep cervical abscess. The Hib vaccination rates, including at least one shot, among cases of IHiD in 2008, 2009, 2010, 2011, 2012, and 2013 were 0%, 0.3% (1/32), 4.2% (2/48), 0%, 28.6% (2/7), and 50.0% (1/2), respectively. The capsular type of isolated *H. influenzae* strains was found for 115 strains (115/141; 81.6%). Of these 115 strains, 107 (93.0%) were Hib strains and 8 (7.0%) were non-typeable *H. influenzae* (NTHi). Yearly changes in Hib coverage in 2008, 2009, 2010, 2011, 2012, and 2013 were 100%, 100%, 89.7%, 92.3%, 66.7%, and 50.0%, respectively (Fig. 2). Among 5 strains isolated from the patients who received at least one shot of Hib conjugate vaccine, 3 strains were Hib and 2 strains were NTHi. All vaccinated patients were <2 years of age.

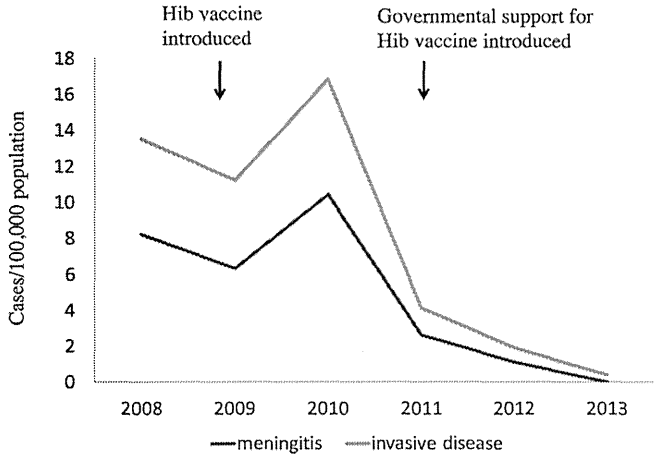

Fig. 1. Yearly change of incidence of invasive *Haemophilus influenzae* disease and meningitis. The highest annual incidence rates of invasive *Haemophilus influenzae* disease (IHiD) and *H. influenzae* meningitis among children less than 5 years of age in the study period were 16.8 and 10.4 per 100,000, respectively, both in 2010; the corresponding lowest annual incidence rates were 0.4 and 0, respectively, both in 2013. This study revealed a 94.7% reduction in IHiD in children younger than 5 years of age from 2010 to 2013 in Chiba prefecture, Japan. This remarkable reduction was observed after the introduction of special funding from the Japanese government for Hib conjugate vaccination.

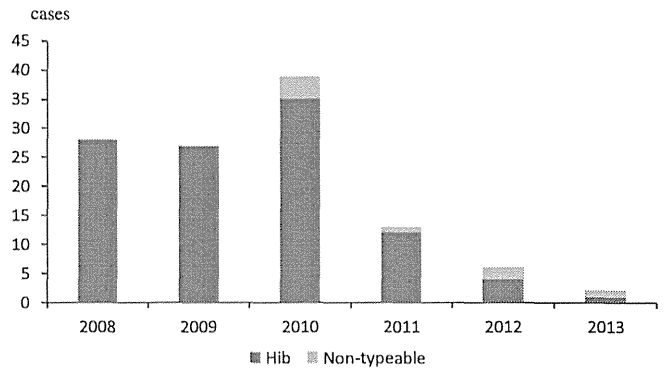

Fig. 2. Number of cases of invasive *Haemophilus influenzae* disease in children, by serotype. The capsular type of isolated *Haemophilus influenzae* strains was found for 115 strains. Of these, 107 (93.0%) were *H. influenzae* type b (Hib), and 8 (7.0%) were non-typeable *H. influenzae* (NTHi). Yearly changes in Hib coverage in 2008, 2009, 2010, 2011, 2012, and 2013 were 100%, 100%, 89.7%, 92.3%, 66.7%, and 50.0%, respectively.

Among 3 patients with invasive Hib disease, 2 patients received one Hib vaccine dose and the remaining 1 received the initial 3 doses. Among 2 patients with invasive NTHi disease, 1 patient received 1 Hib vaccine dose and the other received the initial 3 doses. Overall rates of IHiD and meningitis decreased significantly among the <5 years of age group, with the exception of non-Hib disease, after introduction of governmental support for the Hib vaccine (Table 2). The IRR for all IHiD cases and for invasive vaccine serotypes cases were the same (0.16). This result reflected the fact that the majority of the isolated strains from IHiD cases were Hib.

3.2. IPD

During the 6 study years, 311 patients were diagnosed with IPD. The number of cases of IPD in 2008, 2009, 2010, 2011, 2012 and 2013 was 61, 76, 72, 42, 34 and 26, respectively. Among 311 patients with IPD, 182 patients had bacteremia without a focus, 84 had pneumonia, 33 had meningitis, 9 had cellulitis, 2 had osteomyelitis and 1 had arthritis (Table 3). The highest annual incidence rate of IPD and pneumococcal meningitis among children <5 years of age during the study period were 26.1 (in 2009) and 3.4 (in 2010) per

Table 2
Changes in disease rates by serogroup among patients with invasive *Haemophilus influenzae* disease and invasive pneumococcal disease, 2008–2010 and 2011–2013.

	All cases			Vaccine serotypes			Other than vaccine serotypes		
	2008–2010	2011–2013	*P*	2008–2010	2011–2013	*P*	2008–2010	2011–2013	*P*
Age <5 years, no. of IHiD cases	111	17		85	13		3	2	
Overall	13.8	2.1	<0.05	10.6	2.1	<0.05	0.4	0.3	0.9
IRR (95% CI)	0.16(0.09–0.26)	<0.0001	0.16(0.08–0.28)	<0.0001	0.68(0.05–5.92)	0.67			
Meningitis	8.3	1.2	<0.05	7	1.2	<0.05	0	0	1.0
IRR (95% CI)	0.15(0.06–0.28)	<0.0001	0.18	(0.07–0.33)	<0.0001	NA	NA		
Age <5 years, no. of IPD cases	193	92		47		15		18	31
Overall	24	11.6	<0.05	5.9		1.9	0.15	2.2	4.0
IRR (95% CI)	0.49(0.37–0.63)	<0.0001	0.33(0.17–0.59)	0.0001	1.75(0.95–3.33)	0.055			
Meningitis	2.7	0.9	0.29	1.5	0	0.22	0.3	0.5	0.82
IRR (95% CI)	0.32(0.12–0.79)	0.006	NA	0.0006	2.03	(0.29–22.5)	0.40		

IHiD, invasive *Haemophilus influenzae* disease; IRR, incidence rate ratio; CI, confidence interval.

Table 3
Number of invasive pneumococcal disease cases, by year.

	2008	2009	2010	2011	2012	2013	Total
Bacteremia without focus	30	46	41	30	19	16	182
Pneumonia	24	20	18	8	10	4	84
Meningitis	6	8	10	2	3	4	33
Cellulitis	1	2	3	2	1	0	9
Osteomyelitis	0	0	0	0	0	2	2
Arthritis	0	0	0	0	1	0	1
Total	61	76	72	42	34	26	311
Baseline population[a]	267,191	268.011	268,031	268,670	262,986	256,961	
Incidence[b] (95% CI)	21.3 (16.2–27.6)	26.1 (20.4–33.0)	24.6 (19.0–31.3)	13.8 (9.7–19.0)	11.8 (8.0–16.7)	9.3 (6.0–13.9)	

CI, confidence interval.
[a] Population <5 years of age.
[b] Cases/100,000 population <5 years of age.

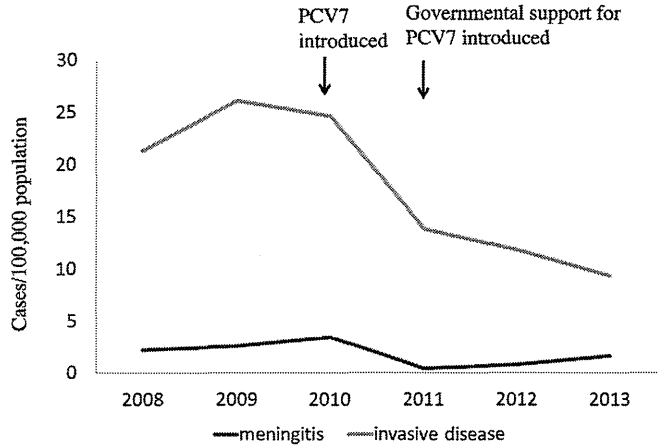

Fig. 3. Yearly change in the incidence of invasive pneumococcal disease and pneumococcal meningitis. The highest annual incidence rates of invasive pneumococcal disease (IPD) and pneumococcal meningitis among children less than 5 years of age in the study period were 26.1 (in 2009) and 3.4 (in 2010) per 100,000, respectively; the corresponding lowest annual incidence rates were 9.3 (in 2013) and 0.4 (in 2011), respectively. This study revealed a 57.4% reduction in IPD in children younger than 5 years of age from 2009 to 2013 in Chiba prefecture, Japan. This remarkable reduction was observed after the introduction of special funding from the Japanese government for heptavalent pneumococcal conjugate vaccination (PCV7).

Table 4
Streptococcus pneumoniae serotype distribution in invasive pneumococcal disease patients.

	2008	2009	2010	2011	2012	2013	Total
6B	4	9	9	3	1	1	27
23F	3	2	4	3	1	0	13
19F	0	4	5	1	0	0	10
14	1	1	3	2	0	0	7
9V	0	1	0	2	1	0	4
4	3	0	0	0	0	0	3
18C	0	0	0	0	0	1	1
19A	1	0	1	2	3	6	13
6A	3	3	0	0	0	1	7
1	1	0	1	0	0	0	2
3	0	0	0	0	1	0	1
7F	0	0	0	0	0	1	1
15A	0	1	0	0	4	1	6
24F	1	1	0	1	0	2	5
15C	0	2	0	1	0	1	4
22F	0	1	0	1	2	0	4
6C	0	1	1	1	0	0	3
38	1	1	0	0	1	0	3
10A	0	0	0	0	1	1	2
33F	0	0	0	0	0	2	2
12F	1	0	0	0	0	0	1
35B	0	0	0	0	1	0	1
Total	19	27	24	17	16	17	120

100,000, respectively; the corresponding lowest annual incidence rates were 9.3 (in 2013) and 0.4 (in 2011), respectively.

The mean annual incidence rates of IPD and pneumococcal meningitis among children less than 5 years of age ranged from 9.3 to 26.1 and 0.4 to 3.4 per 100,000, respectively (Fig. 3). The ages at onset of IPD were as follows: 55 (17.7%) were in the 0 year old subgroup, 133 (42.8%) in the 1 year old subgroup, 97 (31.2%) in the 2–4 year old subgroup, and 26 (8.4%) in the 5 years or older subgroup. Males constituted 58% (181/311) of the subjects. At least one underlying condition was documented in 66 (21.2%) of the 311 patients with IPD. These included malignancy ($n=15$), congenital anomaly/syndrome ($n=9$), neurological disorder ($n=7$), congenital heart disease ($n=8$), bronchial asthma ($n=3$), and others ($n=24$). Among IPD cases occurring at age 5 and over, 69.2% had underlying disease.

Of the 311 study patients, 12 patients (3.9%) developed permanent sequelae, including 10 patients with permanent neurological complications; 9 of these 10 patients had meningitis and 1 had septic shock with bacteremia. A total of 3 patients (1.1%) died, of which 1 was aged 6 months without underlying diseases, 1 was aged 1 year with myopathy, and 1 was aged 1 year with congenital anomaly/syndrome. The PCV7 vaccination rates, including at least one shot, for cases of IPD in 2008, 2009, 2010, 2011, 2012, and 2013 were 0%, 0%, 4.2% (3/72), 9.5% (4/42), 44.1% (15/34), and 65.4% (17/26), respectively. The capsular type of the isolated *S. pneumoniae* strains was found for 120 strains (120/311; 38.6%). Of these 120 strains, 65 (54.2%) were PCV7 serotypes, 24 (20.0%) were PCV13 serotypes (excluding PCV7 serotypes), and 31 (25.8%) were non-PCV13 serotypes (Table 4). The serotyping rate for IPD patients was lower than the rate for those with IHiD (81.6%). The majority of IPD patients had bacteremia. Bacteremia cases were sometimes reported after hospital discharge and so the samples had already been discarded. On the other hand, the majority of IHiD patients had meningitis. Many doctors reported meningitis cases when the definitive diagnosis was made; therefore, it was easy to send us the samples for analysis of serotyping. Yearly changes of PCV7 coverage in 2008, 2009, 2010, 2011, 2012, and 2013 were 57.9%, 63.0%, 87.5%, 64.7%, 18.8%, and 11.8%, respectively. Table 5 shows the number of cases of IPD by number of vaccine doses received, serotype, and patient age. Among 39 vaccinated patients, 14 (35.9%) received 4 doses of PCV7 and 29 (74.4%) were less than 2 years of age. A total of 23 strains were collected from the patients who had at least one shot of PCV7. Of these 23 strains, 7 were serotype 19A, 4 were 15A, 2 were 6B, 15C, 22F, 33F, and 1 was 7F, 10A, 24F, 35B. Overall rates of IPD decreased significantly in the <5 years of age group after introduction of governmental support for PCV7 vaccination. On the other hand, the rate of IPD caused by non-PCV7 serotypes increased, but the change was not statistically significant (Table 2).

4. Discussion

To our knowledge, this is the first report in the English literature that details population-based IHiD and IPD incidence rates in children before and after the introduction of the Hib conjugate and PCV7 vaccines in Japan. In Japan, Hib conjugate vaccine has been available on a voluntary basis since December 2008 and PCV7 since February 2010, but the vaccination rate was estimated to be under 10% in those years. From February 2011, Hib and PCV7 vaccination was encouraged for children under 5 years of age throughout Chiba prefecture by implementation of an official program, the Provisional Special Fund for the Urgent Promotion of Vaccination. This fund was supported by both the national and local governments. The subsidy amount differed among regions in Japan. After the introduction of the special fund, children <5 years of age who lived in all regions of Chiba prefecture could receive these two vaccines free of charge.

As a result, Hib conjugate vaccine and PCV7 immunization rates were estimated to have been more than 50% in 2011 [12]. To our knowledge, there are no official data on immunization rates for these two vaccines in Chiba prefecture. The estimated immunization rate for the two vaccines among infants in Chiba prefecture, based on the volume of product shipped, was more than 80% in 2012.

In the 1980s, Hib conjugate vaccines provided proof of the concept that conjugation of polysaccharide antigen to carrier protein elicits protective antibody formation in infants and toddlers [13]. Following the introduction of Hib conjugate vaccine, Hib disease incidence rates declined with near elimination of invasive Hib disease in many countries where the vaccination is routine for all infants [14–16]. Before the Hib conjugate vaccine era in Japan,