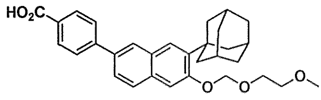
Fig. 7

A



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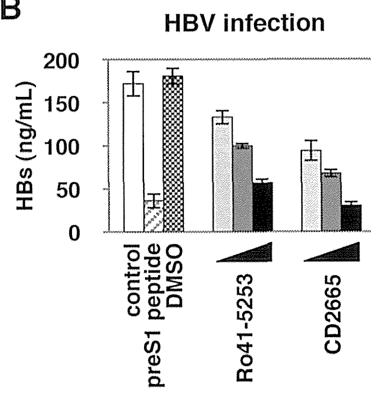
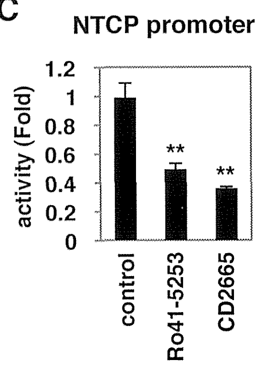


C



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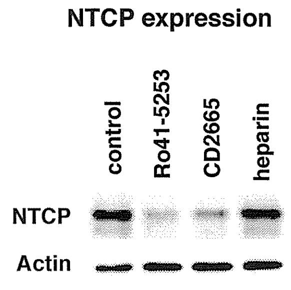


Fig. 8

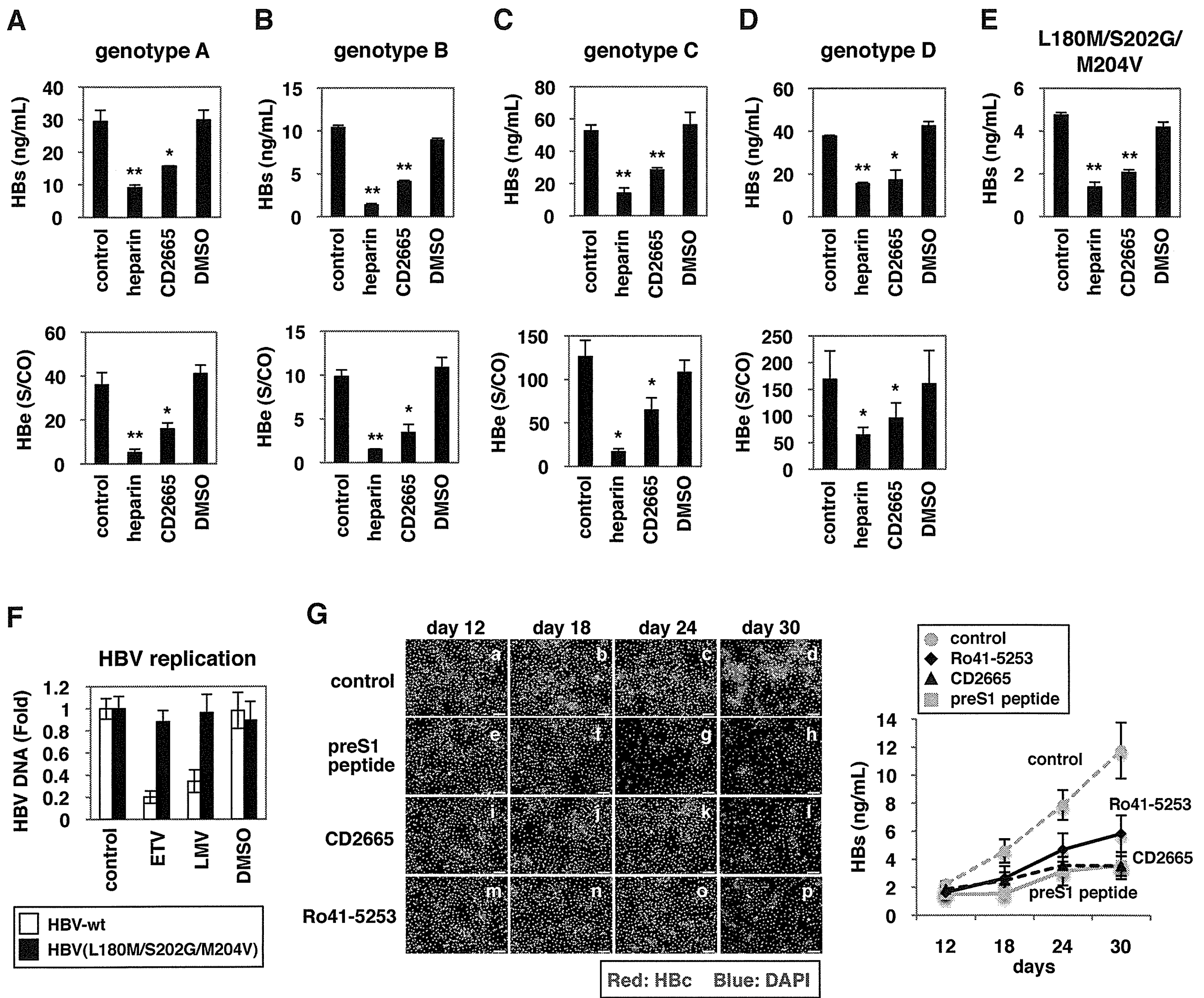
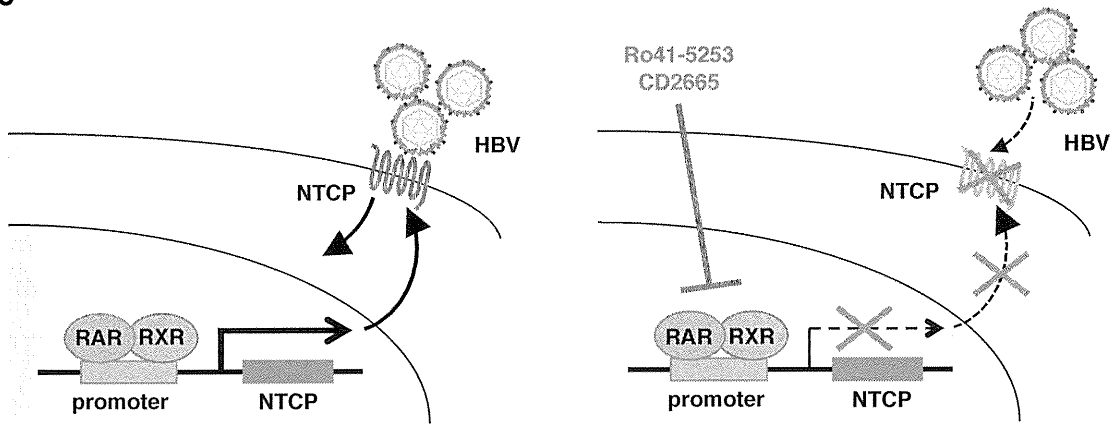


Fig. 9



Chronic Hepatitis B Prevalence among Children and Mothers: Results from a Nationwide, Population-Based Survey in Lao People's Democratic Republic

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Abstract

Background: Hepatitis B is regarded as a serious public health issue in Lao People's Democratic Republic (Lao PDR), a Southeast Asian country. However, disease epidemiology among the general population is not well known, and thus a nationwide cross-sectional survey for hepatitis B surface antigen (HBsAg) prevalence in children and their mothers was conducted.

Methods and findings: We applied three-stage cluster sampling using probability proportionate to size. After randomly selecting child (5 to 9 years old) and mother (15 to 45 years old) pairs from the selected villages, questionnaires and HBsAg rapid tests were conducted. Data from 965 child and mother pairs were analyzed. Multivariate logistic regression analyses were used to investigate the independent association of individual background characteristics for the odds of being HBsAg positive. In total, 17 children and 27 mothers were HBsAg positive. HBsAg prevalence was estimated to be 1.7% (95% confidence interval: 0.8%-2.6%) in children, and 2.9% (95% confidence interval: 1.7%–4.2%) in their mothers after taking sampling design and weight of each sample into account. Mother's infection status was positively associated with HBsAg positivity in children ($p<0.001$), whereas other potential risk factors, such as ethnicity, proximity to health centers, and history of surgery, were not. There were no significant associations between mother's HBsAg status and history of surgery, and other sociodemographic factors.

Conclusions: Despite the slow implementation of the hepatitis B vaccination program, HBsAg prevalence among children and their mothers was not high in Lao PDR compared to reports from neighboring countries. The reasons for the differences in prevalence among these countries are unclear. We recommend that prevalence surveys be conducted in populations born before and after the implementation of a hepatitis B vaccination program to better understand the epidemiology of hepatitis B.

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Introduction

More than two billion people have been infected with hepatitis B worldwide, and among these individuals, more than 350 million suffer from chronic hepatitis B virus (HBV) infection [1,2,3]. Infection with HBV results in 600,000 to 1.2 million deaths per year due to chronic hepatitis, cirrhosis, and hepatocellular carcinoma [2,4]. HBV is responsible for 60% to 80% of the world's hepatocellular carcinoma cases, one of the major three causes of death in Africa, Asia, and the Pacific Rim, and accordingly, has been categorized as a Group 1 carcinogenic agent to humans by the International Agency for Research on Cancer [5].

The prevalence of hepatitis B differs throughout the world. Southeast Asian countries have been estimated to have a chronic HBV infection rate of more than 8% before the introduction of hepatitis B vaccination [6]. The Western Pacific region of the World Health Organization (WHO), to which most of the Southeast Asian countries belong, is assumed to have a high prevalence of hepatitis B [7]. Specifically, the prevalence is estimated to be 9% to 12% among women of childbearing age [8] and 8% to 10% among children in pre-vaccine era [9]. The WHO

estimates that the region has 28% of the global population, while it accounts for almost half of all chronic hepatitis B infections worldwide [10].

Hepatitis B vaccination, especially within 24 hours after childbirth, is considered the most effective and efficient preventive measure against hepatitis B infection [3,11]. Based on these assumptions, the WHO set goals to lower the prevalence of chronic hepatitis B among children over 5 years of age to 2% by 2012 and 1% by 2017. To achieve these goals, the WHO plans to increase immunization coverage to 65% for the birth dose and 80% for the third dose of the hepatitis B vaccine [7].

Lao People's Democratic Republic (Lao PDR) is a Southeast Asian country, located in the center of the Indochina peninsula. The country is landlocked and surrounded by China, Vietnam, Cambodia, Thailand, and Myanmar. The neighboring countries report relatively high hepatitis B prevalence compared to other parts of the world. For example, a survey from two provinces in Cambodia reported a hepatitis B surface antigen (HBsAg) prevalence of 7.7% (95% CI: 6.2%–9.3%) among healthy volunteer adults [12]. Another population-based survey in a province in rural Vietnam found that 18.8% (95% CI: 15.7%–21.9%) of adults and 12.5% (95% CI: 9.7%–15.3%) of infants were HBsAg positive at the time of the survey [13]. Thus, Lao PDR has been regarded as one of the hyperendemic countries for hepatitis B for quite some time and is ranked as a priority country by the WHO [7,9] despite a lack of data on the prevalence in a representative population. Pre-vaccine era prevalence was estimated as 11.8% [4], 8–10% [9], or 8% or more [6] for Lao PDR and Indochina countries. In response to this situation, Lao PDR has implemented the hepatitis B vaccine into the routine immunization program since 2002 (at 6, 10, and 14 weeks after birth), as well as birth dosing since 2004. The birth dosing was initiated at referral hospitals in the capital city, and then gradually expanded into rural hospitals (2006), and eventually home deliveries (2010). However, since then, no direct investigation has been conducted, and thus a nationwide survey is warranted [7,9]. The routine immunization coverage is reported as 56% for BCG, 50% for the third DPT, 50% for the third hepatitis B, 40% for measles, and 46% for oral polio vaccine in 2007, when a proportion of target children were born [14].

The primary objective of the present study was to estimate the chronic HBV infection rates by measuring the seroprevalence of HBsAg among children aged 5 to 9 years, and their mothers aged 15 to 45 years.

Methods

Ethical considerations

The survey protocol was reviewed and approved by the Ethical Committee of the Ministry of Health, Lao PDR, and the institutional review board of the National Center for Global Health and Medicine, Japan (NCGM-G-001130-00). Access to selected households was granted by the Ministry of Health, and the provincial and district government authorities.

After obtaining approval to conduct the survey from local authorities, surveyors explained the purpose of the survey to village leaders, selected participants, and their caregivers, assured them that all information would be strictly confidential and that no names would be gathered, and that there would be no benefit or penalties for agreeing or refusing to participate. Written informed consent was obtained from each mother on behalf of her child for each pair. Written informed consent was obtained from legal representatives (next of kin, caregivers, or guardians) when mothers were illiterate. The respondents' names were not recorded on the questionnaire sheets.

Study population

The target population was children aged 5 to 9 years (date of birth: January 2, 2002 to January 1, 2007) and their mothers aged 15 to 45 years (date of birth: January 2, 1966 to January 1, 1997) living in the selected cluster at the time of the survey. The reasons for this selection criteria are: 1) the national and regional hepatitis control policy target is to reduce chronic hepatitis B prevalence among children aged 5 years or older [7]; 2) Lao PDR does not have reliable HBsAg prevalence data among healthy adults, and mothers of childbearing age are considered the major source of hepatitis B infection for children; and 3) our pilot survey revealed that between 20 and 25 mother and child pairs can be practically sampled from each village.

Calculation of sample size

The equation used to calculate the required sample size is as follows [15,16]:

$$n = Z^2 \times p(1-p)DEFF \times 2/(d^2 \times RR)$$

where n = sample size
Z = significance level for 95% confidence
p = expected prevalence
$DEFF$ = design effect
d = precision
RR = response rate

The sample size (n) of 961 was calculated on the basis of an expected HBsAg seroprevalence (p) of 5%, a 5% level of significance (Z), precision (d) of ±2.0%, design effect ($DEFF$) of 2.0, two strata, and response rate (RR) of 95%. For field practicability, we requested 24 survey teams to sample 21 child and mother pairs from each cluster, with the aim of gathering 1,008 pairs in total.

Survey design and sampling

The survey applied a stratified three-stage random cluster sampling design, a type of probability sampling recommended by the WHO [15,17]. The survey was carried out by 24 survey teams (two members per team). Team members were recruited from the same districts that were under investigation to implement the survey more smoothly. The survey teams consisted of epidemiology, surveillance, or laboratory staff. The survey teams were supervised by 11 national personnel (six from the National Immunization Program and five from the National Center for Laboratory and Epidemiology, Ministry of Health) as well as 13 provincial officers.

For stratified multistage cluster sampling, immunization coverage by district and population data were obtained from the National Immunization Program, the Ministry of Health, and the Department of Statistics, Lao PDR. For post-survey weight adjustment, the survey teams obtained the latest population data from village leaders or health volunteers.

All 143 districts in Lao PDR were stratified into two strata, one having high (more than 76%) and the other having low (76% or less) immunization coverage for the third diphtheria, pertussis, tetanus, and hepatitis B (DPT-HepB)vaccines as reported in 2010. For the first stage, we selected 12 districts from each stratum using probability proportionate to size (PPS) sampling based on the population census of 2005. For the second stage, we selected two villages from each selected district by PPS sampling, and 48

villages were randomly sampled in total. In the instances in which the selected village lacked a sufficient number of children or the survey team could not approach the selected village due to safety or security reasons, the nearest village on the way back to the district center was selected. For each selected village, surveyors obtained a list of households, including age and sex, primarily from the poverty reduction program data with the assistance of the village leader, women's union, and/or healthcare volunteer. From these lists, 21 mothers aged 15 to 45 years old with children aged 5 to 9 years were randomly selected using a paper-based lottery system. When a mother had multiple children aged 5 to 9 years old, the youngest child was chosen for the survey. Special attention was paid to ensure that the child's biological mother was surveyed, as adoption is common in rural Lao PDR.

The survey was carried out from January 25th to February 4th, 2012. Each survey team successfully approached their assigned villages, with the exception of one village, which could not be visited because of road difficulties. An alternative village was chosen according to the predetermined selection criteria. In total, 1,008 children and 1,008 mothers were sampled. The overall response rate for HBsAg was 100%; however, 43 pairs were excluded from the analysis due to age ineligibility. That is, one child was over 9 years of age and 33 were less than 5 years of age. Furthermore, three mothers were over 45 years of age and six were less than 15 years of age. This happened as 43 mothers confused calendar age with traditional age. In rural areas, newborns start at one year old and a year is added to their age for each passing of a Lunar New Year. The surveyors asked participants for their age in years and their date of birth, and checked that they matched. A total of 965 pairs were included for analysis.

Questionnaires

A brief face-to-face questionnaire was administered to the sampled mother. The questionnaire consisted of 25 questions in four domains of inquiry: sociodemographic background of the family (i.e., ethnicity, family head's occupation, and mother's education level), family history of liver diseases, including mother, demographic characteristics of the child (i.e., age, sex, and place of birth), and immunization records. Additionally, questions were asked regarding exposure to potential risk factors for acquiring hepatitis B infection (e.g., history of blood transfusion, surgical operation, and sharing of toothbrush). The questionnaire was developed in English, translated into Lao, back-translated into English, and then compared and revised by bilingual staff members. A small pilot test was conducted prior to the data collection.

Testing for HBsAg

We used a simple and rapid test (Alere Determine HBsAg test card; Alere Medical Co. Ltd., Chiba, Japan) rather than the traditional ELISA test, as it was better suited to use in the field [14]. The sensitivity and specificity of the test were reported as high in two Asian countries [18,19]. In Vietnam, the Determine HBsAg test validity was measured based on comparison with HBsAg EIA. Results were 100% in both sensitivity and specificity in 328 samples [18]. In China, the Determine HBsAg performance was evaluated in comparison with HBsAg EIA for 671 samples. The sensitivity was reported to be 98.9% and specificity 100% [19]. The Determine HBsAg examination kit is one of the most reliable point-of-care HBsAg tests, and is recommended by the WHO [15]. HBsAg testing was performed according to the manufacturer's instructions. Blood was collected from a finger prick using a safety lancet (BD Safety Lancet, Becton Dickinson, NJ, USA) and glass capillary tube, and the blood was applied onto the sample pad of the rapid test kit. After applying the chase buffer, surveyors assessed the results after at least 15 minutes, but no longer than 24 hours. When no control bar appeared after 15 minutes, the test results were considered invalid, and the test was repeated. Blood spots were collected onto filter paper for further testing. A 2-day training session was organized for surveyors and supervisors on the use of the rapid test and the completion of the questionnaire. To ensure the safety of the blood collection procedure, surveyors always used a new pair of latex gloves. Surveyors were instructed to place all capillary tubes and lancets into safety boxes immediately after use.

Data entry and statistical analysis

All of the completed questionnaires were brought to a centralized location and the data were entered into a Microsoft Excel 2007 spreadsheet. Data were double-entered and cross-checked. Logistic regression tests and odds ratios were used to examine the relationship between the independent variables and HBsAg results. Multivariate logistic regression was used to investigate the independent association of different household and individual characteristics with the odds of being HBsAg positive. All estimates and standard errors were calculated by taking the multistage clustered sampling design and the weight of each sample into account to give representative, unbiased results. A p value <0.05 was considered statistically significant.

In our regression analyses, we adjusted for potential confounders by using the following variables: third DPT-HepB immunization coverage at the location of current residence, mother's age, ethnic group, mother's education level, family head's occupation, and mother's HBsAg status. For multivariate logistic regression analyses, multicollinearity was tested by calculating the variance inflation factors for each independent variable, and a value of more than 10 was considered as having multicollinearity.

All statistical analyses were carried out using STATA version 12 (Stata Corp., College Station, TX). Means and proportions were calculated using STATA's 'svy' function, with each sample weighted according to estimated population size.

Results

Socioeconomic backgrounds

The baseline characteristics of the 965 mothers and their children are summarized in Table 1. The mean age of the mothers was 29.1 years (95% CI: 26.2–33.1), and the mean age of the children was 5.8 years (95% CI: 5.4–6.3). Of the sampled children, 474 (49.4%) were male and 486 (50.6%) were female (five were unknown).

HBsAg prevalence among the general population

Of the 965 pairs included in the study, 17 children and 27 mothers were positive for HBsAg. Six child and mother pairs were HBsAg positive. The estimated prevalence was 1.7% for children (95% CI: 0.8%–2.6%) and 2.9% for mothers (95% CI: 1.7%–4.2%) after taking the sampling design and weight of each sample into account. HBsAg prevalence did not change significantly between DPT-HepB3 high and low coverage districts in both children and mothers (Table 2).

Potential risk factors

To determine whether background characteristics affect HBsAg status, we conducted multivariate logistic regression analysis in children and their mothers. In children, the mother's HBsAg status was positively associated with hepatitis B infection (Table 3),

Table 1. HBsAg prevalence among children (5 to 9 years old) and mothers (15 to 45 years old) in Lao PDR by selected background characteristics.

		n	%	Children's HBsAg (+)	%	95% CI	Mothers' HBsAg (+)	%	95% CI
Mothers' age(n = 965)	15–19	4	0.41	0	0.00		0	0.00	
	20–24	85	8.80	1	1.18	0.00–3.52	3	3.53	0.00–7.53
	25–29	294	30.47	7	2.38	0.63–4.13	8	2.72	0.85–4.59
	30–34	275	28.50	6	2.18	0.44–3.92	9	3.27	1.16–5.39
	35–39	176	18.24	3	1.70	0.00–3.64	3	1.70	0.00–3.64
	40–45	131	13.58	0	0.00		4	3.05	0.07–6.04
Ethnicity (n = 963)	Low land Lao	651	67.60	9	1.38	0.48–2.28	19	2.92	1.62–4.22
	Mid land Lao	248	25.75	6	2.42	0.49–4.34	5	2.02	0.25–3.78
	High land Lao	64	6.65	2	3.13	0.00–7.51	3	4.69	0.00–10.01
[1]Transportation (n = 939)	on foot	298	31.74	1	0.34	0.00–1.00	6	2.01	0.41–3.62
	bicycle	14	1.49	0	0.00		0	0.00	
	motor bike	364	38.76	7	1.92	0.51–3.34	10	2.75	1.06–4.43
	car	183	19.49	5	2.73	0.35–5.12	6	3.28	0.67–5.88
	hand tractor	66	7.03	3	4.55	0.00–9.71	4	6.06	0.15–11.97
	other	14	1.49	0	0.00		0	0.00	
[2]Time (n = 901)	< 5 minutes	31	3.44	0	0.00		1	3.23	0.00–9.81
	5 to 15 minutes	274	30.41	3	1.09	0.15–2.33	6	2.19	0.45–3.93
	15 to 30 minutes	231	25.64	5	2.16	0.27–4.06	11	4.76	2.00–7.53
	30 to 60 minutes	209	23.20	5	2.39	0.30–4.48	4	1.91	0.04–3.79
	> 60 minutes	156	17.31	3	1.56	0.00–4.68	4	2.56	0.06–5.07
[3]Education (n = 962)	did not finish primary school	307	31.91	7	2.28	0.60–3.96	12	3.91	1.73–6.09
	primary school	374	38.88	5	1.34	0.17–2.51	10	2.67	1.03–4.32
	junior high	185	19.23	3	1.62	0.00–3.46	2	1.08	0.00–2.59
	high school	73	7.59	0	0.00		1	1.37	0.00–4.10
	college/univ	20	2.08	1	5.00	0.00–15.47	2	10.00	0.00–24.41
	other or unknown	3	0.31	1	33.33	0.00–100.00	0	0.00	
[4]Occupation (n = 963)	farmer	683	70.92	13	1.90	0.88–2.93	19	2.78	1.55–4.02
	fisherman	5	0.52	0	0.00		0	0.00	
	laborer	92	9.55	1	1.09	0.00–3.25	5	5.43	0.71–10.16
	public officer	88	9.14	1	1.14	0.00–3.40	3	6.25	1.70–10.80
	factory employee	8	0.83	0	0.00		0	0.00	
	general employee	16	1.66	1	6.25	0.00–19.57	0	0.00	
	merchant	63	6.54	1	1.59	0.00–4.76	0	0.00	
	others	8	0.83	0	0.00		0	0.00	
Mother's surgery (n = 962)	yes	95	9.88	2	2.11	0.00–5.05	3	3.16	0.00–6.74
	no	867	90.12	15	1.73	0.86–2.60	24	2.77	1.67–3.86
Child's sex (n = 960)	male	474	49.38	9	1.89	0.67–3.13			
	female	486	50.63	7	1.44	0.38–2.50			
Place of delivery (n = 961)	province hospital	207	21.54	4	1.93	0.04–3.82	6	2.90	0.59–5.20
	district hospital	105	10.93	2	1.90	0.00–4.56	5	4.76	0.62–8.90
	health center	10	1.04	0	0.00		0	0.00	
	private clinic	11	1.14	0	0.00		1	9.09	0.00–29.35
	at home	569	59.21	8	1.41	0.44–2.38	14	2.46	1.18–3.74
	in the forest	56	5.83	3	5.36	0.00–11.44	1	1.79	0.00–5.36
	other health facility	3	0.32	0	0.00		0	0.00	
Child's surgery (n = 960)	yes	22	2.29	0	0.00				

Table 1. Cont.

	n	%	Children's HBsAg (+)	%	95% CI	Mothers' HBsAg (+)	%	95% CI
no	938	97.71	16	1.71	0.88–2.54			

[1]Transportation to the nearest health facility, [2] Time to the nearest health facility, [3] Mothers' completed education, [4] Family head's occupation.
doi:10.1371/journal.pone.0088829.t001

whereas the other potential risk factors were not associated according to the adjusted odds ratio. We did not obtain information regarding the type of delivery, and we did not find significant differences in HBsAg prevalence associated with delivery settings. No independent factor was positively associated with HBsAg positivity in mothers, according to the adjusted odds ratio (Table 4).

Immunization status

Written immunization records were available for 213 out of 965 children (22.1%). One hundred ninety eight children were vaccinated with three doses of hepatitis B vaccine, and 34 children were immunized on the day of birth or the following day. Five out of 213 children with immunization records were HBsAg positive (2.35%; 95% CI: 0.30–4.40%), while 12 of 752 without immunization records were HBsAg positive (1.60%; 95% CI: 0.70–2.49%). The differences between the two groups were not significant ($p = 0.46$).

Discussion

HBsAg prevalence among the general population

The estimated HBsAg prevalence in the general population was much lower in both children and adults than that of previous reports from neighboring countries and Lao PDR. For example, HBsAg prevalence in adults in Cambodia, Thailand, and Vietnam was reported to be 7.7% (95% CI: 6.2%–9.3%) [12], 6 to 10% [15,20], and 18.8% (95% CI: 15.7%–21.9%) [13], respectively. Data on HBsAg prevalence amongst children was relatively scarce, and reported to be 3.5% (95% CI: 2.4%–4.8%) in Cambodia [21], and 18.4% (95% CI: 13.4%–23.4%) in Vietnam [13]. In Lao PDR, studies in blood donors, hospitalized patients, and Lao migrant workers tested in Thailand showed HBsAg prevalence of 8.73% (95% CI: 8.69%–8.77%) [22], 17.99% (95% CI: 17.81%–18.17%) [23], and 6.86% (95% CI: 6.80%–6.92%) [24] based on the given numerators and denominators in the articles, respectively.

Since the study objective was to estimate the nationwide HBsAg prevalence among the general population of Lao PDR, and thus the study design is a cross sectional survey, it is difficult to explain the reasons for the unexpectedly low prevalence. There are several potential explanations for this observation. The survey methodology used was very different from that used for blood donors, patients, and migrant workers. We used probability sampling and thus the results are representative of the whole population, whereas studies of blood donors, hospitalized patients, and migrant workers used non-probability sampling and therefore the results are restricted to these populations. The primary objective of our survey was to estimate HBsAg prevalence among the general population, so probability sampling was the most appropriate choice. Demographic conditions among the sampled population are determined by survey methodology, and therefore the results showed discrepancy. The WHO strongly recommends probability sampling for hepatitis B prevalence survey [7,15,17]. Although Lao PDR has the lowest population density of the Indochina peninsula countries [25], the precise effects on hepatitis B prevalence of the reduced frequency of human to human contact due to the country's relatively low population density and less developed infrastructure remain unclear.

The number of HBsAg positives varied from 0 to 4 per cluster. Since the sampling design of the survey aimed to estimate the prevalence in the whole country, it is difficult to determine whether these differences reflect the local endemic status.

Potential risk factors

Our survey revealed that no potential risk factors were significantly associated with the children's infection status, with the exception of the mothers' hepatitis B infection status. HBsAg prevalence surveys in other countries revealed that history of surgery [26,27], level of education [26], and ethnicity [28] were independently associated with hepatitis B infection. The reason why we could not find any potential risk factors positively associated with hepatitis B infection among children is not clear. However, it should be noted that the primary objective of the present study was to assess HBsAg prevalence, and not its risk factors. Additionally, some reports found that HIV positive individuals are positively associated with hepatitis B virus infection

Table 2. HBsAg prevalence among children (5 to 9 years old) and mothers (15 to 45 years old).

	Children's HBsAg (+)	%	95% CI	Standard error	Design effect	Mothers' HBsAg (+)	%	95% CI	Standard error	Design effect
High coverage districts (n = 486)	6	1.14	0.23–2.04	0.44	0.82	18	3.79	1.79–5.79	0.97	1.24
Low coverage districts (n = 479)	11	2.39	0.75–4.03	0.79	1.27	9	1.88	0.49–3.37	0.69	1.22
Total (n = 965)	17	1.72	0.81–2.63	0.44	1.10	27	2.93	1.65–4.20	0.61	1.28

doi:10.1371/journal.pone.0088829.t002

Table 3. Unadjusted and adjusted odds ratio for being HBsAg positive among children from five to nine years old in Lao PDR by selected background characteristics.

		Unadjusted odds ratio	95% CI	p	Adjusted odds ratio	95% CI	p
DPT3 coverage	high	1(reference)					
	low	2.13	0.73–6.21	0.16	3.47	0.77–15.64	0.10
Mothers' age	15 to 29	1(reference)					
	30 to 45	0.70	0.28–1.78	0.44	0.87	0.31–2.47	0.79
Ethnicity	Low land Lao	1(reference)					
	others	1.90	0.67–5.40	0.22	1.41	0.26–7.72	0.68
Education	none	1(reference)					
	finished primary school or upper	1.50	0.67–3.36	0.30	1.03	0.27–3.89	0.96
Occupation	white collar	1(reference)					
	blue collar	1.15	0.37–3.64	0.80	0.60	0.18–1.96	0.38
Sex	male	1(reference)					
	female	0.75	0.21–2.62	0.63	0.65	0.21–2.08	0.46
Birth place	health facility	1(reference)					
	non-health facility	0.98	0.39–2.49	0.97	0.79	0.28–2.21	0.64
Mothers' HBsAg	negative	1(reference)					
	positive	24.02	9.45–61.07	0.00	28.13	10.21–77.53	0.00

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[29,30]; however, we did not investigate HIV due to limited budget.

WHO's regional target

The interim target of the WHO is to reduce HBsAg prevalence to less than 2% in children aged at least 5 years old by 2012 [7,31]. The point prevalence is used for monitoring the control of hepatitis B. The Regional Office for the Western pacific recommended that the country conduct a national HBsAg prevalence survey to verify whether the country has reached the regional prevalence target [9]. Following these criteria, Lao PDR had already achieved its goal. However, it is unlikely that Lao PDR achieved the target through the immunization program alone because the country has the lowest immunization coverage of all countries in the region [7,9]. Considering the relatively lower HBsAg seroprevalence among the mothers compared to those reported in previous studies, it is likely that Lao PDR had a lower prevalence even before the introduction of the hepatitis B immunization program. Therefore, the final target of reducing HBsAg prevalence to less than 1% in children aged at least 5 years could be difficult to achieve if the country simply continues its current immunization policy.

A nationwide prevalence survey targeting the general population is ideally conducted before implementing the immunization

Table 4. Unadjusted and adjusted odds ratio for being HBsAg positive among mothers from 15 to 45 years old in Lao PDR by selected background characteristics.

		Unadjusted odds ratio	95% CI	p	Adjusted odds ratio	95% CI	p
DPT3 coverage	high	1(reference)					
	low	0.50	0.20–1.28	0.14	0.47	0.19–1.16	0.10
Mothers' age	15 to 29	1(reference)					
	30 to 45	1.03	0.43–2.51	0.94	0.94	0.39–2.25	0.88
Ethnicity	Low land Lao	1(reference)					
	others	0.80	0.30–2.17	0.65	0.68	0.25–1.85	0.44
Education	none	1(reference)					
	finished primary school or upper	1.68	0.70–4.01	0.23	2.04	0.89–4.68	0.09
Occupation	white collar	1(reference)					
	blue collar	1.71	0.53–5.55	0.35	1.93	0.68–5.50	0.21
History of surgery	no	1(reference)					
	yes	1.28	0.39–4.25	0.67	1.30	0.35–4.78	0.68

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strategy to evaluate hepatitis B epidemiology. However, we were able to understand the epidemiology to some degree, even after implementation of immunization policy, because adults usually represent the pre-vaccination era [15,17].

Strengths of the study

The present study is the first nationwide survey on the prevalence of hepatitis B in the general population both before and after the implementation of a hepatitis B immunization policy in Lao PDR and other Southeast Asian countries. We applied multistage stratified cluster sampling to better represent the general population. The design effect of prevalence was calculated between 0.8 and 1.3, which was acceptable as we set it around 2.0 before the survey.

The background characteristics of our sampled population were similar to those of another nationwide population-based study, the Lao PDR Reproductive Health Survey (LRHS) [32] conducted in 2005. For example, the locations of current residence (north, central, and south) were 33.3%, 41.7%, and 25.0% in our survey, and 38.6%, 38.9%, and 22.5% in the LRHS. The levels of mothers' completed education (none, primary school, secondary school or more) were 31.9%, 38.9%, and 29.2% in our survey, and 28.8%, 43.7%, and 27.5% in the LRHS. The LRHS applied the multistage stratified cluster sampling method and surveyed more than 13,000 women all over the country. A direct comparison of the populations sampled by the two different surveys is difficult to perform as the primary objectives were different. Despite this, our sampled population is considered to likely represent the general population in Lao PDR.

Limitations of the study

There are several limitations in our study that should be addressed. First, the population data is based on the census conducted in 2005. After 2005, the population distribution may have changed and some of the villages could have merged, thereby creating bias in the findings. Fortunately, we did not survey any villages that disappeared or merged.

Second, floating or marginal populations are likely to be missed from the residential lists, and these populations could be a source of HIV and hepatitis B virus infections [33]. In future seroprevalence surveys, these subpopulations should be accounted for by using specific approaches, such as oversampling.

Third, population immunity levels were difficult to measure or estimate. The possession of immunization certificates was low, because many participants had already finished their scheduled vaccinations before 12 months of age, and relevant documents were lost. In the present study, we did not have enough data from health centers due to time and budget limitations. Since we did not examine immunization markers, such as HBsAb, herd immunity levels are unknown.

Lastly, adult men were not included in the survey. Serological studies in the past indicated that men have higher HBsAg rates than women [8,21,28]. In Lao PDR, male blood donors presented with 9.7% HBsAg positive prevalence, while the prevalence in females was 6.2% [22]. When considering the disease burden of hepatitis B virus infections, it is better to include both sexes [26].

To the best of our knowledge, this is the first nationwide, population-based serological survey on chronic hepatitis B virus infections both before and after implementation of hepatitis B immunization in Southeast Asia, where disease burden is high. As such, our results provide valuable information on a hepatitis B immunization program and a useful baseline against which to compare future assessments in this region.

National immunization policy should be based on the disease epidemiology [3]. However, in Southeast Asia, understanding of the epidemiology of hepatitis B remains unsatisfactory. Even when a country implements a hepatitis B immunization program for children and the prevalence of disease reaches the target (i.e., less than 2% among children aged 5 years or older), we cannot conclude that the immunization program alone contributed to reduced disease prevalence without comparing it to the disease prevalence in the pre-vaccine generation, i.e., adults. Nationwide surveys assessing disease prevalence in the generations before and after the implementation of a vaccination program will provide valuable information for understanding hepatitis B epidemiology. Therefore, we recommend surveying hepatitis B seroprevalence in both generations.

Conclusions

We determined the nationwide HBsAg prevalence among children (1.7%; 95% CI: 0.8%–2.6%) and their mothers (2.9%; 95% CI: 1.6%–4.2%) in Lao PDR. This is the first report to estimate the nationwide prevalence of chronic hepatitis B in pre- and post-hepatitis B immunization generations in Southeast Asia, where hepatitis B infections are a substantial burden. The estimated prevalence was below that of previous studies, suggesting that our understanding of this disease's epidemiology is lacking and warrants further investigation. We recommend that the prevalence among the pre- and post-vaccine eras should be investigated when conducting hepatitis B seroprevalence surveys.

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Author Contributions

Conceived and designed the experiments: AX MH KI TW MS. Performed the experiments: KK TK PV CP KP DP BS VSO TS. Analyzed the data: KK TK MH. Contributed reagents/materials/analysis tools: KI TW MS. Wrote the paper: AX PV MH. Revised the manuscript: KK TK PV CP KP DP BS VSO KI TW MS. Arranged laboratory for diagnosis: PV KI TW MS.

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CASE REPORT

Acute hepatitis B of genotype H resulting in persistent infection

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Abstract

A 47-year-old man presented with general fatigue and dark urine. The laboratory data showed increased levels of hepatic transaminases. The patient was positive for hepatitis B virus (HBV) markers and negative for anti-human immunodeficiency virus. The HBV-DNA titer was set to 7.7 log copies/mL. The patient was diagnosed with acute hepatitis B. The HBV infection route was obscure. The serum levels of hepatic transaminases decreased to normal ranges without any treatment, but the HBV-DNA status was maintained for at least 26 mo, indicating the presence of persistent infection. We isolated HBV from the acute-phase serum and determined the genome sequence. A phylogenetic analysis revealed that the isolated HBV was genotype H. In this patient, the elevated peak level of HBV-DNA and the risk alleles at human genome single nucleotide polymorphisms s3077 and rs9277535 in the human leukocyte antigen-DP locus were considered to be risk factors for chronic infection. This case suggests that there is a risk of persistent infection by HBV genotype H following acute hepatitis; further cases of HBV genotype H infection must be identified and characterized. Thus, the complete determination of the HBV genotype may be essential during routine clinical care of acute hepatitis B outpatients.

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Key words: Acute hepatitis; Chronic hepatitis; Genotyping; Hepatitis B virus; Single nucleotide polymorphisms

Core tip: Hepatitis B virus (HBV) genotype H infection is rare in Asia, particularly in Japan. Here, we report a case of acute hepatitis B caused by a genotype H strain with persistent infection, although most adult cases of acute hepatitis B are self-limiting in Japan. This case suggests that the HBV genotype H infection can be a risk factor for persistent infection. Therefore, it is necessary to investigate the characteristics of genotype H infection in an accumulation of cases. Thus, the

complete determination of the HBV genotype may be essential in the routine clinical care of acute hepatitis B patients.

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INTRODUCTION

Hepatitis B is a potentially life-threatening liver infection caused by the hepatitis B virus (HBV); it represents a major global health problem. HBV can cause chronic liver diseases and increases the risk of death from cirrhosis and liver cancer. Worldwide, an estimated two billion people have been infected with HBV and more than 240 million have chronic infections[1]. The HBV genome consists of approximately 3200-nucleotides of DNA; the virus replicates using a reverse transcriptase enzyme that lacks proofreading ability. Therefore, HBV possesses diverse genetic variability, and the viral population is classified into at least eight genotypes that are designated A -H[2-6]. In Japan, genotypes B and C are prevalent among patients with chronic infections. However, in the last decades, the prevalent genotype in acute HBV infections has shifted from genotype C to A[7-9]. There are some differences in the clinical features and outcomes among the genotypes[10-13]. It has been reported that the persistent infection from acute hepatitis is prevalent in adults that are infected with genotype A HBV. Thus, determining the HBV genotype is of increasing importance even in routine clinical practice, although a reliable kit for determination of all HBV genotypes is still uncommon and is not yet covered by insurance. The host factors associated with persistent infection by HBV have also been reported, such as single nucleotide polymorphisms (SNPs) or genotypes in the human leukocyte antigen-DP locus. It may also be useful for identifying the patients who are prone to develop chronic hepatitis.

In this report, we describe a case of acute hepatitis B resulting from infection by a genotype H strain of HBV. Although the laboratory data and symptoms were not distinguishable from acute hepatitis B with other genotypes, this patient developed persistent infection.

CASE REPORT

A 47-year-old man living in Kawasaki, Japan, presented at our hospital with general fatigue and dark urine. Approximately 1 wk before visiting the hospital, the patient developed nausea, loss of appetite, and a feeling of fullness in the abdomen. Four days later, he noted darkening of his skin and urine. Upon admission, the patient's laboratory data revealed elevated serum aspartate aminotransferase, alanine aminotransferase (ALT), lactate dehydrogenase, alkaline phosphatase, γ-glutamylt ranspeptidase, and total bilirubin (T-Bil) levels (Table 1). The prothrombin activity was within the normal range (95%). Test for hepatitis B surface antigen (HBsAg; HISCL-2000i, Sysmex, Kobe, Hyogo, Japan), hepatitis B e-antigen (HBeAg; ARCHITECT® CLIA, Abbott Japan, Tokyo, Japan) and anti-hepatitis B core antigen (anti-HBc) IgM (ARCHITECT® CLIA) were positive. A test for HBV-DNA was also positive, exhibiting a titer of 7.7 log copies/mL (COBAS TaqMan HBV Test v2.0, Roche Diagnostics, Tokyo, Japan). HBsAg had not been detected 2 years previously when the patient had been admitted to another hospital for treatment of acute enterocolitis. Other hepatitis virus markers were negative. Therefore, the patient was diagnosed with acute hepatitis B. The genotype of the infecting HBV, as assessed by the Immunis HBV Genotype Immunis® HBV Genotype EIA Kit (Institute of Immunology, Tokyo, Japan), was determined as genotype C. The patient had not been abroad in the past 12 mo; he had no history of receiving blood or blood-related products, transfusions, or drug injections, and he reported no personal or family history of liver disease. The man was unmarried and declared that he was heterosexual, with no history of sexual contact with commercial sex workers or strangers. Anti-human immunodeficiency virus (HIV) was not detected. In the absence of medication, the patient's condition and elevated ALT level improved within a month. Anti-HBe became detectable, and HBeAg disappeared 2 mo after onset of the symptoms. HBsAg became undetectable at 5 mo, but the patient still tested positive for HBV-DNA, a status that persisted for at least 26 mo following his presentation at our hospital (Figure 1). We are now preparing to administer anti-viral medication.

For further analysis of the HBV infecting this patient, HBV-DNA was extracted from the acute-phase serum using a QIAamp DNA Blood Mini kit (QIAGEN, Valencia, CA). The entire HBV genome sequence was determined after polymerase chain reaction (PCR) amplification using the following primers [the number of nucleotides (nt) added to the primers were deduced from the prototype HBV/C clone, with accession no. AB246344]. For the amplification of half of the HBV genome, the outer primers were 5'-ATTCCACCAAGCTCTGCTAGATCCCAGAGT-3' (nt 10-39) and 5'-GGTGCTGGTGAACAGACCAATTTATGCCTA-3' (nt 1813-1784), and the inner primers were 5'-CCTATATTTTCCTGCTGGTGGCTCCAGTTC-3' (nt 46-75) and 5'-TAGCCTAATCTCCTCCC CCAACTCCTCCCA-3' (nt 1760-1731). For the other half of the HBV genome, the outer primers were 5'- ACGTCGCATGGAGACCACCGTGAACGCCCA-3' (nt 1601-1630) and 5'-AAGTCCACCACGAGTCTAGACTCTGTGGTA-3' (nt 266-237), and the inner primers were 5'-CCAGGTCTTGCCCAAGGTCTTACATAAGAG-3' (nt 1631-1660) and 5'-CCCGCCTGTAACACGAGCAGGGGTCCTAGG-3' (nt 207-178). The PCR was performed in a thermal cycler for 30 cycles

Table 1 Laboratory findings at first visit to our hospital

Hematology		Blood chemistry		Viral markers		Immunology		Coagulation	
WBC	7400/μL	TP	7.4 g/dL	Anti-HA IgM	(-)	IgA	183 mg/dL	PT%	95%
Neutrophil	72.0%	Albumin	4.5 g/dL	Anti-HCV	(-)	IgG	1168 mg/dL	APTT	36.4 s
Eosinophil	1.0%	T-Bil	11.1 mg/dL	HBsAg	(+) 197333	IgM	220 mg/dL		
Basophil	0.0%	D-Bil	8.0 mg/dL	Anti-HBc IgM	(+) 25.5 C.O.I	ANA	× 40, homogeneous		
Monocyte	10.0%	AST	1942 IU/L	HBeAg	(+) 253 C.O.I				
Lymphocyte	17.0%	ALT	2963 IU/L	Anti-HBe	(-) 0.0 %				
RBC	457/μL	ALP	612 IU/L	HBV-DNA	7.7 log copies/mL				
Hemoglobin	16.0 g/dL	γGTP	756 IU/L	Anti-HIV	(-)				
Hematocrit	46.4%	LDH	739 IU/L	RPR	(-)				
Platelet	36.6 × 10^4/μL	BUN	8.2 mg/dL	TPHA	(+)				
		Creatinine	0.64 mg/dL	Anti-CMV IgG	(+)				
		T-Chol	225 mg/dL	Anti-CMV IgM	(-)				
				Anti-EBV EBNA	(+)				
				Anti-EBV EA IgG	(-)				
				Anti-EBV VCA IgG	(+)				
				Anti-EBV VCA IgM	(-)				

WBC: White blood cells; RBC: Red blood cells; ANA: Antinuclear antibody; TP: Total protein; T-Bil: Total bilirubin; D-Bil: Direct bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; γGTP: γ-glutamyltranspeptidase; LDH: Lactase dehydrogenase; BUN: Blood urea nitrogen; T-Chol: Total cholesterol; PT: Prothrombin activity; APTT: Activated partial thromboplastin time; C.O.I: Cutoff index; HA: Hepatitis A; HCV: Hepatitis C virus; HBsAg: Hepatitis B surface antigen; HBc: Hepatitis B core; HBeAg: Hepatitis B e-antigen; HBV: Hepatitis B virus; HIV: Human immunodeficiency virus; RPR: Rapid plasma regain; TPHA: Treponema pallidum hemagglutination assay; CMV: Cytomegalovirus; EBV: Epstein-Barr virus; EBNA: Epstein-Barr virus nuclear antigen; EA: Early antigen; VCA: Viral capsid antigen.

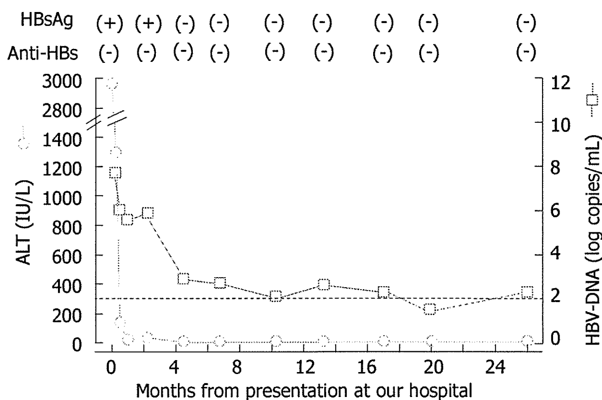

Figure 1 Clinical course of the patient infected with the genotype H strain. The dotted line indicates the detection limit of HBV-DNA (2.1 log copy/mL); the titer of the HBV-DNA was below the lower limit at 18 mo. HBsAg: Hepatitis B surface antigen; Anti-HBs: Antibody to hepatitis B surface antigen; ALT: Alanine aminotransferase; HBV: Hepatitis B virus.

(94 ℃, 30 s; 60 ℃, 30 s; 72 ℃, 30 s) with TAKARA LA Taq® DNA polymerase (TAKARA, Shiga, Japan). The amplified fragments were sequenced directly with an automated DNA sequencer (3500 Genetic Analyzer, Applied Biosystems, Foster City, CA, United States).

The genome of the infecting HBV (designated as B-MHJ9014) was 3215 bases in size. A phylogenetic analysis was performed with this strain and several database reference strains. B-MHJ9014 sorted with the genotype-H branch of the tree and clustered with the genotype-H strains previously isolated from Japanese patients (Figure 2). The substitutions at nt 1762 and nt 1764 (the basal core promoter region) and at nt 1896 (the precore region) were not observed. The length of the deduced amino acid sequences of the S, X, Core, and P proteins were identical to those encoded by other genotype H strains in the databases. The α determinant region of the S protein of B-MHJ9014 harbored an amino acid polymorphism (phenylalanine to leucine) at residue 134. The predicted B-MHJ9014 reverse transcriptase did not include any of the amino acid substitutions known to be associated with nucleotide analog resistance. To assess the complexity of the infecting virus, S region sequences from 51 clones in acute phase serum were determined. The detected sequences were genotype H and were closely related to the consensus sequence determine by direct sequencing with 1-3 amino acids polymorphisms (data not shown).

To assess the presence of human genome SNPs in the HLA-DP locus that are associated with persistent infection by HBV[14,15], a blood specimen was obtained from the patient (who had previously provided informed consent). Genomic DNA was extracted from buffy coat samples with the QIAamp DNA Mini kit (QIAGEN); DNA for SNPs rs3077 and rs9277535 were amplified with the appropriate primers and TAKARA LA Taq® DNA polymerase and were sequenced directly. The patient was homozygous (G/G) at both of these SNPs; these alleles are considered to be risk alleles for persistent infection.

DISCUSSION

HBV genotype H was first reported in 2002[5]. Infections by this genotype have been found mainly in Nicaragua, Mexico, and California; this genotype is considered to be rare in Asia, particularly in Japan[5,16-18]. However, since the first recognition of genotype H in Japan in 2005, eight strains have been isolated from Japanese patients (Table 2)[18-25]. All reported genotype H strains were isolated from male patients aged 35 to 65 years old, and the major route of infection was sexual transmission (5/8, 62.5%). Four cases (50%) represent transmissions that

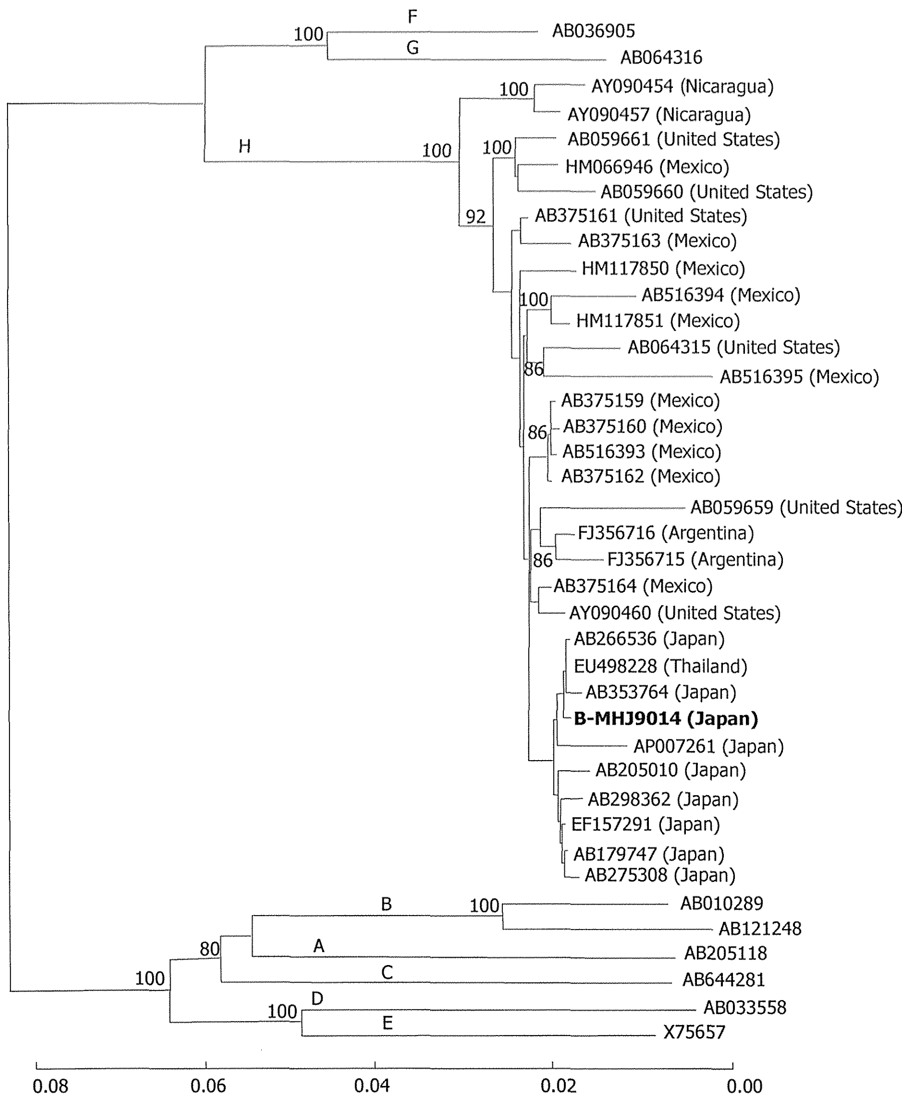

Figure 2 A phylogenetic trees constructed using the neighbor-joining method with the full hepatitis B virus genome sequence of the isolated and reference strains. The strain isolated in this case (B-MHJ9014) is shown in bold. The horizontal bar indicates the number of nucleotide substitutions per site. The reference sequences are shown with the DDBJ/EMBL/GenBank accession numbers. The HBV genotypes are indicated on each branch. The bootstrap values (> 80%) are indicated at the nodes as a percentage of the data obtained from 1000 resamplings. HBV: Hepatitis B virus.

Table 2 Genotype H strains reported in Japan

No.	Patient		Hypothesized source of infection		HIV infection[1]	Clinical feature	Accession number (Ref.)
	Age	Gender	Route	Place			
1	52	Male	Unknown	Japan	NA	Unknown blood donor	AB179747, [18]
2	61	Male	Sexual contact (heterosexual)	Thailand	NA	Chronic	AB205010, [19]
3	46	Male	Sexual contact (bisexual)	South America	(+)	Chronic	AP007261, [20]
4	38	Male	Sexual contact (homosexual)	Unknown	NA	Chronic	AB298362, [21]
5	65	Male	Unknown	Japan	NA	Acute	EF157291, [22]
6	35	Male	Unknown	Japan	NA	Acute	AB266536, [23]
7	60	Male	Sexual contact (homosexual)	Japan	(-)	Acute	AB275308, [24]
8	60	Male	Sexual contact (heterosexual)	Unknown	(+)	Chronic	AB353764, [25]
9	47	Male	Unknown	Japan	(-)	Acute to chronic	AB846650, this paper

[1]NA: Not available; HIV: Human immunodeficiency virus.

occurred in Japan. Co-infection with HIV was not common (2/8, 25%). These characteristics were similar to the case described here. All isolated strains from Japanese patients clustered together as a branch on the phylogenetic tree; therefore, it is possible that a specific strain of genotype H has emerged and spread in Japan. Presumably, the infrequent use of a reliable and convenient detection kit for genotype H infection has hampered the

correct diagnosis of genotype H infection; some cases may be misdiagnosed and considered to be infections by other genotypes. In fact, in the current case, our HBV isolate was originally identified as genotype C by the commercial kit that is covered by insurance in Japan. This kit was developed before the discovery of genotype H; thus, such a misidentification is a potential risk, as noted in the kit's instruction manual. The clinical features of genotype H infection remain obscure. There is a growing need for an accumulation of genotype H infection cases. To this end, the use of a reliable HBV genotyping kit that can correctly distinguish all genotypes is essential for routine clinical practice.

In Japan, most cases of acute hepatitis B are self-limiting, but some cases have been reported to have progressed to persistent infections[9,26-29]. Among the reported cases of genotype H infection, 4 strains were isolated from chronic hepatitis patients; in all cases, the infection was ascribed to sexual contact (Table 2)[19-21,25]. In our case, the HBV-DNA persisted for at least 26 mo. To our knowledge, this report represents the only case of genotype H infection in which chronic hepatitis was observed following acute infection. HBsAg was no longer detected at 4 mo from onset by HISCL-2000i. This disappearance was also confirmed by ARCHITECT® HBsAg (CMIA, Abbott Japan, Tokyo, Japan). In the S protein analysis, we found an amino acid polymorphism in the α determinant region. This polymorphism may affect the sensitivity for detecting HBsAg. HIV infection, a well-known risk factor for prolonged HBV infection[30], was not detected in our patient. Recently, the risk factors for HBV persistent infection have been reported in an analysis of a cohort that excluded patients co-infected with HIV[29]. In that report, infection with genotype A, elevated peak levels of HBV-DNA, and attenuated peak levels of ALT were suggested as risk factors for chronic infection. In the case described here, the peak level of HBV-DNA was 7.7 log copy/mL, which was consistent with increased risk for chronic infection. However, our patient exhibited a peak level of ALT of 2963 IU/L, which is a value that would classify this individual in the self-limiting group. Therefore, the clinical features of this case did not completely fit the risk factors associated with the establishment of chronic infection in the previous analysis[29]. Another reported risk factor for chronic HBV infection is the presence of certain SNP alleles. Specifically, selected SNPs around the HLA-DP locus have been reported to be associated with chronic hepatitis B in Asians[14,15]. With the informed consent of our patient, we determined the sequences for these SNPs (rs3077 and rs9277535) and found that this patient harbored risk alleles at both polymorphisms. This factor may have contributed to the establishment of chronic infection in this case.

In conclusion, we report a case of acute hepatitis B caused by a genotype H strain of HBV. This patient exhibited persistent infection. Our finding suggests that the infection of HBV genotype H can be a risk factor for persistent infection. We believe that it is necessary to use kits that are capable of accurate genotyping to permit an accumulation of cases and to investigate the clinical features of genotype H infection in routine clinical practice.

COMMENTS

Case characteristics

The main symptoms were nausea, loss of appetite, and a feeling of fullness in the abdomen.

Clinical diagnosis

The patient was a case of acute hepatitis B caused by a genotype H strain with persistent infection.

Differential diagnosis

The hepatitis B virus (HBV) genotype was considered to be important to predict the outcome and clinical features.

Laboratory diagnosis

To diagnose this patient, the detection of HBV markers and the complete determination of the HBV genotype were essential.

Treatment

The anti-viral treatment was not administered because we expected this case was self-limiting. Authors are now preparing medication.

Experiences and lessons

The infection of HBV genotype H can be a risk factor for persistent infection and the complete determination of HBV genotype is important.

Peer review

To conclude the association between HBV genotype H and chronic infection, the accumulation of cases of genotype H infection is essential.

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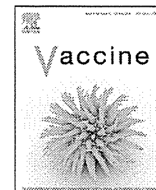

Evaluation of single-round infectious, chimeric dengue type 1 virus as an antigen for dengue functional antibody assays

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ABSTRACT

Dengue fever and dengue hemorrhagic fever are endemic throughout tropical and subtropical countries. Four serotypes of dengue viruses (DENV-1 to DENV-4), each with several genotypes including various subclades, are co-distributed in most endemic areas. Infection–neutralizing and -enhancing antibodies are believed to play protective and pathogenic roles, respectively. Measurement of these functional antibodies against a variety of viral strains is thus important for evaluating coverage and safety of dengue vaccine candidates. Although transportation of live virus materials beyond national borders is increasingly limited, this difficulty may be overcome using biotechnology that enables generation of an antibody-assay antigen equivalent to authentic virus based on viral sequence information. A rapid system to produce flavivirus single-round infectious particles (SRIPs) was recently developed using a Japanese encephalitis virus (JEV) subgenomic replicon plasmid. This system allows production of chimeric SRIPs that have surface proteins of other flaviviruses. In the present study, SRIPs of DENV-1 (D1-SRIPs) were evaluated as an antigen for functional antibody assays. Inclusion of the whole mature capsid gene of JEV into the replicon plasmid provided higher D1-SRIP yields than did its exclusion in cases where a DENV-1 surface-protein-expressing plasmid was used for co-transfection of 293T cells with the replicon plasmid. In an assay to measure the balance between neutralizing and enhancing activities, dose (antibody dilution)-dependent activity curves in dengue-immune human sera or mouse monoclonal antibodies obtained using D1-SRIP antigen were equivalent to those obtained using DENV-1 antigen. Similar results were obtained using additional DENV-2 and DENV-3 systems. In a conventional Vero-cell neutralization test, a significant correlation was shown between antibody titers obtained using D1-SRIP and DENV-1 antigens. These results demonstrate the utility of D1-SRIPs as an alternative antigen to authentic DENV-1 in functional antibody assays. SRIP antigens may contribute to dengue vaccine candidate evaluation, understanding of dengue pathogenesis, and development of serodiagnostic systems.

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1. Introduction

Dengue fever (DF) and dengue hemorrhagic fever (DHF) are the most globally important mosquito-borne viral diseases [1,2]. The World Health Organization estimates that 50–100 million infections, including 500,000 DHF cases and approximately 12,500 deaths, occur annually [3]. Vaccines and specific antivirals are currently unavailable. DF and DHF are caused by any of four types of dengue viruses (DENVs) generally designated as serotypes DENV-1 to DENV-4. All of these serotypes are currently co-distributed in most tropical and subtropical areas worldwide [4]. Additionally, each of the four serotypes has 4–6 distinct genotypes with subclades that are locally distributed in various areas and countries [5]. Moreover, introduction of foreign DENV strains occurs in many areas, sometimes accompanied by increases in the number of patients or higher proportions of severe cases (DHF) [6–11]. Furthermore, a new DENV serotype genetically and serologically distinct from the current four serotypes has also recently been discovered [12]. Potential thus exists for human exposure to a variety of DENV strains.

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Several dengue vaccine candidates have been developed and are currently being evaluated in clinical trials [13,14]. All of these candidates are able to induce neutralizing antibody in humans. Neutralizing antibody contributes to reduction in viremia levels and is believed to be an important factor in disease protection [15]. Most neutralizing antibody species against DENVs display infection-enhancing activity in sub-neutralizing doses in vitro [16]; there is consequent concern that neutralizing antibody-inducible dengue vaccine may cause antibody-dependent enhancement (ADE) of infection if insufficient neutralizing antibody levels are induced by vaccination [17,18]. ADE, the mechanism most likely responsible for increased viremia levels, is a process in which monocytes are efficiently infected in an Fc gamma receptor (FcγR)-mediated manner [19]. Measurement of vaccine-induced neutralizing and enhancing antibodies is therefore important for dengue vaccine evaluation. Because of their potential human infectivity, various DENV strains are required as antigens in the antibody assays.

Despite these requirements, transportation of live virus materials beyond national borders is increasingly limited owing to current regulations, such as governmental security export control policies [20] as well as access and benefit-sharing restrictions of the Convention on Biological Diversity [21]. These limitations may be overcome using biotechnology that enables the generation of material equivalent to the authentic virus based on viral full-genome nucleotide sequence information. Although the technique to construct an infectious clone of DENV has already been established [22–25], the method is arduous and thus not practical for preparation of various antigens for antibody assays.

A novel system to generate flavivirus single-round infectious particles (SRIPs) has recently been established [26]. This method exploits a Japanese encephalitis virus (JEV) subgenomic replicon plasmid lacking coding regions of capsid (C), pre-membrane (prM), and envelope (E) structural proteins. SRIPs are produced by co-transfection of this replicon plasmid with a plasmid expressing JEV structural proteins into 293T cells. As a DNA-based production system, this method facilitates simple and rapid antigen generation. Most importantly, chimeric SRIPs have also been produced using a plasmid expressing structural proteins of other flaviviruses (e.g., dengue, yellow fever, and tick-borne encephalitis viruses), although production levels of chimeric SRIPs derived from DENVs have been much lower than those derived from JEV and other flaviviruses. The successful production of flavivirus SRIPs in this system suggests its potential utility for functional antibody assays, as SRIP surface antigens can be theoretically designed based on prM and E coding region nucleotide sequences.

The purpose of the present study was to evaluate the utility of DENV-1 SRIPs (D1-SRIPs) as an antigen for neutralizing and enhancing antibody assays. Results of assays using dengue-immune human serum samples or mouse monoclonal antibodies (MAbs) against DENV-1 demonstrated that antibody levels obtained using D1-SRIP antigen were equivalent to those obtained using authentic DENV-1 antigen. These results indicate that D1-SRIPs can serve as an alternative functional antibody assay antigen to DENV-1. The use of DENV antigens in the form of SRIPs for neutralizing and enhancing antibody assays may thus be suitable for dengue vaccine candidate evaluation.

2. Materials and methods

2.1. Cells

Human embryonic kidney 293T cells (CRL-3216; American Type Culture Collection [ATCC], Manassas, VA) were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Vero, C6/36, and K562 cells and their culture media have been described previously [27]. All cells were cultivated in a humidified atmosphere of 5% CO_2–95% air at 37 °C, except for C6/36 cells, which were cultivated at 28 °C.

2.2. Viruses

The Mochizuki strain of DENV-1, New Guinea C (NGC) strain of DENV-2, and H87 strain of DENV-3 were used [28]. Culture fluids harvested from infected C6/36 cells were used as viral antigens in neutralization tests and in an assay to measure the balance between neutralizing and enhancing antibodies.

2.3. Antibodies

Human serum samples previously collected from general patients aged 29–71 years in Indonesia during 1999–2001 and stored at −20 °C [29] were used as antibody specimens for evaluating SRIP antigen. Each of these sera had detectable neutralizing antibody titers against all four DENV serotypes (Supplementary Table 1). As a negative control, we used a human serum sample collected from a residence in a non-dengue-endemic country (Japan) that showed no detectable neutralizing activities against any DENV serotypes. Heat inactivation of sera was performed at 56 °C for 30 min. The use of human serum samples was approved by the Ethical Committee of the Faculty of Tropical Medicine, Mahidol University. MAbs specific for DENV-1 (D1-IV-7F4) or crossreactive to all DENV serotypes (D1-III-9B1, D1-IV-3B8, D1-V-3H12, D1-V-8E8 [30], and JE-10B4 [31]) and D1-4G2 (E-specific, flavivirus group-crossreactive; HB-112, ATCC) in an ascites form were also used for evaluating SRIP antigen. Mouse MAb JE-2D5, specific for JEV non-structural protein 1 (NS1) [32], was used for immunostaining.

2.4. Plasmids

Plasmids pCMV-JErep, pCAG-JEC [26], pcD1ME, pcD2ME, and pcD3ME [28] have been described previously (Fig. 1A). Briefly, pCMV-JErep is a JEV replicon plasmid designed to transcribe viral RNA in transfected cells, and is the full genome of JEV Nakayama strain (GenBank no. EF571853) except lacking 2238 nucleotides (positions 150–2387) corresponding to main portions of C and E and the entire prM gene. pCAG-JEC is an expression plasmid for JEV (Nakayama) mature C, consisting of 105 amino acids, while pcD1ME, pcD2ME, and pcD3ME are expression plasmids for prM and E of DENV-1 (Mochizuki), DENV-2 (NGC), and DENV-3 (H87), respectively. In this study, a portion of C in pCMV-JErep was replaced by the full JEV mature-C region to construct the new replicon plasmid pCMV-JErep-fullC, which was consequently lacking 1971 nucleotides (positions 438–2408) corresponding to a portion of C not responsible for synthesis of mature C, the full prM and a major portion of E (Fig. 1A).

2.5. Preparation of SRIPs

293T cells in a 6-well-plate well were co-transfected with 1 μg each of two plasmids, pCMV-JErep-fullC and pcD1ME (Set II of Fig. 1A), using Lipofectamine LTX and Plus reagent (Invitrogen, Gaithersburg, MD) following the manufacturer's instructions. Culture fluids harvested on days 3–7 served as D1-SRIP antigen in neutralization tests using Vero cells and in an assay to measure the balance between neutralizing and enhancing antibodies using K562 cells. For titration of D1-SRIPs on K562 cells, serial dilutions of D1-SRIPs (50 μl/well) prepared in 96-well poly-L-lysine-coated plates were mixed with 5×10^4 semi-adherent K562 cells (50 μl/well). The mixture was incubated at 37 °C for 2 days, followed by fixation and immunochemical staining (see below). Infective titers

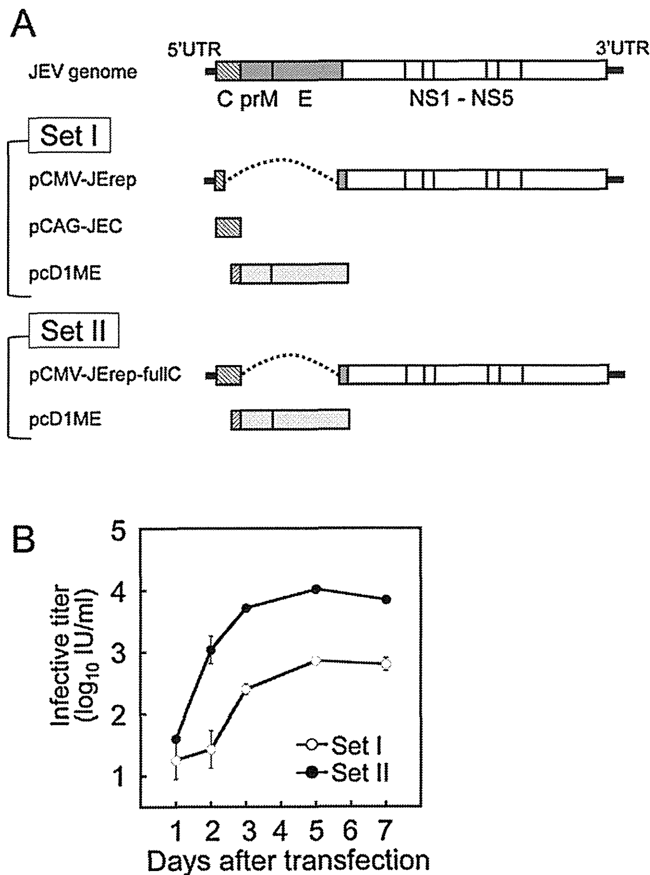

Fig. 1. Generation of D1-SRIPs. (A) Schematic diagram of the JEV genome and DENV-1 genes used to generate D1-SRIPs. pCMV-JErep, pCAG-JEC, and pCMV-JErep-fullC were constructed based on the JEV genome, while pcD1ME was based on DENV-1 genes. Dotted lines show portions deleted from the JEV genome: nucleotide positions 150–2387 for pCMV-JErep and 438–2408 for pCMV-JErep-fullC. Sets I and II refer to combinations of plasmids used for transfection of 293T cells. (B) Time course of D1-SRIP yield. 293T cells grown in 6-well plates were co-transfected with 1 μg of each plasmid in Sets I (3 μg total; open circles) or II (2 μg total; closed circles), and culture fluids were harvested 1, 2, 3, 5, and 7 days after transfection. Infective titers in the culture fluids were measured using K562 cells. Each datum represents an average of values obtained in three wells, with SDs indicated by bars.

were determined by counting the number of infected cells and were expressed as infectious units per ml (IU/ml). SRIPs of DENV-2 (D2-SRIPs) and DENV-3 (D3-SRIPs) were prepared and titrated essentially by the same method used for D1-SRIPs.

2.6. Immunostaining

Immunochemical staining of Vero cells and semiadherent K562 cells was performed essentially as described previously [33]. Briefly, fixed cells were serially incubated with primary antibody (mouse MAb), biotinylated anti-mouse IgG, avidin–biotinylated peroxidase complex (ABC) reagents, and VIP substrate (Vector Laboratories, Burlingame, CA). To determine DENV-infected cells, D1-4G2 was used as a primary antibody to stain DENV E antigen. To determine SRIP-infected cells where no dengue antigens were produced, JE-2D5 was used to stain JEV NS1 antigen produced in cells infected with SRIPs containing all non-structural protein genes of JEV.

2.7. Measurement of the balance between neutralizing and enhancing activities

The balance between neutralizing and enhancing activities was measured using semi-adherent K562 cells essentially in the same way as described previously [33]. Briefly, serial dilutions of antibody specimens (36 μl/well) prepared in 96-well poly-L-lysine-coated plates were mixed with D1-SRIP or DENV-1 antigens (50 μl/well) in the absence or presence of rabbit complement at a final concentration of 5%. Equivalent infectious titers of D1-SRIPs (2×10^3 IU/ml) and DENV-1 (2×10^3 FFU/ml) were used. The virus-antibody mixture was incubated at 37 °C for 2 h and then mixed with 5×10^4 K562 cells (50 μl/well). The virus-antibody-cell mixture was incubated at 37 °C for 2 (for DENV-1) or 3 (for D1-SRIPs) days. After fixation and immunochemical staining, foci (for DENV-1) or infected cells (for D1-SRIPs) were counted under a microscope. Cut-off values for neutralizing and enhancing activities were calculated from the mean plus or minus three times the standard deviation (SD) obtained using eight negative controls adjusted for approximately 1×10^2 infected cells. Assays measuring neutralizing and enhancing antibody balance using DENV-2, DENV-3, D2-SRIP, and D3-SRIP antigens were performed essentially by the same method as described above for DENV-1 and D1-SRIP antigens.

2.8. Neutralization tests

Conventional Vero-cell plaque reduction neutralization tests were performed with DENV-1 essentially as described previously [28], except for the use of a 96-well plate. Serial dilutions of antibody specimens (36 μl/well) prepared in 96-well plates were mixed with D1-SRIP or DENV-1 antigens (50 μl/well) with or without 5% rabbit complement and incubated at 37 °C for 2 h. The virus-antibody mixture was incubated at 37 °C for 2 h and then mixed with 2.5×10^4 Vero cells (50 μl/well). The virus-antibody-cell mixture was incubated at 37 °C for 3 days, fixed, and immunochemically stained. Neutralizing activity was expressed as the percent reduction in plaque number compared with the control without an antibody specimen. Neutralization tests using D1-SRIP antigen were performed exactly by the same method as those using DENV-1 antigen, excepting that the number of infected cells was used for calculating the percent reduction. The antibody dilution showing a 50% reduction in the number of plaques (for DENV-1 antigen) or infected cells (for D1-SRIP antigen) was obtained using the FORECAST function in Microsoft Excel and expressed as PRNT50.

3. Results

3.1. Increased D1-SRIP yield using a modified replicon plasmid

In our previous study [26], chimeric D1-SRIPs were produced using pCAG-JEC expressing JEV C and pcD1ME expressing DENV-1 prM-E to complement a replicon plasmid lacking C-prM-E genes, pCMV-JErep (Set I in Fig. 1A). However, the yield of D1-SRIPs was too low for use in neutralization tests and assays to measure the balance between neutralizing and enhancing antibodies. To seek higher yields, a new replicon plasmid (pCMV-JErep-fullC) was constructed. This plasmid contained the whole mature C gene of JEV and allowed us to produce D1-SRIPs solely by complementation with pcD1ME (Set II in Fig. 1A). Sets I and II were consequently used for transfection of 293T cells and comparison of D1-SRIP yields.

In both Sets I and II, the highest infective titer was detected in culture fluid 5 days after transfection. The titer obtained with Set II ($\approx 10^4$ IU/ml) was higher than that obtained with Set I ($\approx 10^3$ IU/ml; Fig. 1B). This result suggested that the incorporation of the entire JEV C gene into the replicon plasmid increased yields of chimeric D1-SRIPs. Transfection with Set II gave yields of more than 5.2×10^3 IU/ml on days 3, 5 and 7. Culture fluids harvested 3–7 days after the Set II transfection were therefore used for subsequent evaluations. The single-round infection nature of D1-SRIPs