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Original Article

A seroepidemiological survey of the effect of hepatitis B vaccine and hepatitis B and C virus infections among elementary school students in Siem Reap province, Cambodia

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Aim: This study aimed to survey the prevalence and incidence of hepatitis B (HBV) and hepatitis C virus (HCV) infection among elementary school students in Siem Reap province, Cambodia and to evaluate the effects of a national infant HBV vaccination program introduced in 2001.

Methods: Students in 3rd grade during the 2011, 2012, and 2013 academic years were enrolled in this study; at the time of the second examination, in the 2014–2015 academic year, the students were in 5th or 6th grade. The incidence and prevalence rates of HBV and HCV infection were estimated and full HBV sequences were analyzed.

Results: Among 248 students (107 male and 141 female) born between 1999 and 2005, five students were HBV surface antigen (HBs-Ag) positive (2.02%), and all of them were infected with genotype C. Among them, subgenotype C1 was found in four students and, unexpectedly, complete genetic sequence identity of subgenotype C1 was found in two students from different families. The anti-HBV core (HBc) and anti-HBs prevalence rates were 10.89% and 16.13%, respectively. Twenty-five students were positive for anti-HBs and negative for both HBsAg and anti-HBc (10.08%; estimated serological vaccination rate); this rate increased significantly with the birth year ($P = 0.0229$). Prevalence of anti-HCV was 2.82%, and HCV RNA was not detected. The estimated incidence of HBV and HCV infection were both 0/1000 person-years (PY) (95% confidence interval, 0–20.61/1000 PY and 0–14.50/1000 PY, respectively).

Conclusion: Hepatitis B virus full-genome sequencing and serological analysis revealed the possibility of horizontal transmission of HBV among Cambodian schoolchildren. However, the anti-HBc positivity rate decreased along with increasing age and estimated serological vaccination rates.

Key words: Cambodia, elementary school students, HB vaccination, hepatitis B virus, hepatitis C virus, seroepidemiology

INTRODUCTION

Hepatitis B virus (HBV) and hepatitis C virus (HCV) infections can lead to serious global health problems of hepatocellular carcinoma (HCC) and liver cirrhosis. According to the World Health Organization, an estimated 257 million people worldwide lived with chronic HBV infection in 2015, whereas 71 million people lived with chronic HCV.[1] Furthermore, approximately 600 000 and 350 000 people die from HBV- and HCV-related diseases each year, respectively.[2,3]

The prevalence rates of hepatitis B surface antigen (HBsAg) and HCV-RNA among Cambodian adults are 4.6–7.7% and 2.3%, respectively.[4,5] According to Globocan 2012, liver cancer is the most common cause of cancer-related deaths in Cambodia.[6] As previous reports

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indicated that 75.6% of patients with HCC are infected with HBV and/or HCV,[7] it is important to determine the current health status concerned with HBV and HCV infection in Cambodia.

A national HB vaccination program for infants was introduced in Cambodia in 2001. Hepatitis B virus vaccines are given three times to all infants at the age of 6 weeks, 10 weeks, and 14 weeks with an interval of 4 weeks between each dose. A birth dose (BD) was added to the schedule.[8,9] Currently, data regarding the prevalence of HBsAg among infants born after the implementation of this national vaccination program are limited,[9] and no reports have addressed HBV infection, including hepatitis B core (anti-HBc) and anti-HBs prevalence among this cohort.

We undertook a seroepidemiological survey of Sasar Sdam elementary school students in the Siem Reap province of Cambodia during five periods between 2011 and 2015 with the aims of evaluating the HBV and HCV infection rates and evaluating the effect of the national HB vaccination program among elementary school students.

METHODS

STUDENTS AT THE Sasar Sdam elementary school in Siem Reap province, Cambodia participated in this study from 2011 to 2015. The subjects of this survey were 3rd grade students at their first examination during the 2011, 2012, and 2013 academic years, then they were 5th or 6th grade students at their second examination in the 2014–2015 academic year.

Subjects

The initial prevalence rates of HBsAg, anti-HBc, anti-HBs, HBV DNA, HCV antibody (anti-HCV), and HCV RNA were evaluated among the students. Additionally, students who participated more than once were included in an incidence study (Fig. 1). Prior to the survey, we informed guardians about the significance of this survey and its procedures through interpreters, and the guardians provided written informed consent to participate. Students were given an explanation of the purpose of this survey and the procedure used to collect blood. Subsequently, we obtained informed consent to participate from each student.

Questionnaire

With the Khmer version, the guardians filled out a six-item questionnaire, followed by a section that asked for demographic information (age, birth year, and sex). The items were designed to obtain information about potential risk factors, including the present health condition, history of hospitalization, HB vaccination status, history of surgery and/or blood transfusions, tattoos, and holes for pierced earrings.

Serological tests

After collecting 10 mL blood from each student, samples were centrifuged in the laboratory of Siem Reap Provincial Hospital. Serum samples were stored at 4°C and transferred to Hiroshima University (Hiroshima, Japan) for HBV and HCV infection analysis.

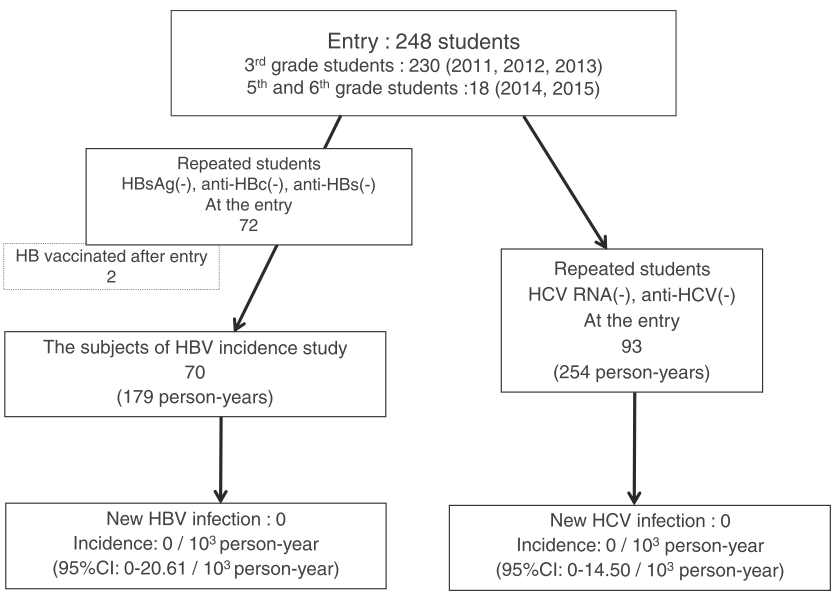

Figure 1 Study of the incidence of hepatitis B virus (HBV) and hepatitis C virus (HCV) infections among elementary school students in Siem Reap, Cambodia.

To investigate HBV infections, HBsAg was detected using reversed passive hemagglutination (Mycell II HBsAg; Institute of Immunology, Tokyo Japan), Chemiluminescent immunoassay (CLIA) (Architect HBsAg QT; Abbott, Tokyo, Japan), and chemiluminescent enzyme immunoassay (CLEIA) (Lumipulse; Fujirebio, Tokyo, Japan), which have respective cut-off points of 2^2, 0.05 IU/mL, and 1.0 (Cut-off index [COI]). Anti-HBc was detected using passive hemagglutination (PHA) (Mycell anti-rHBc; Institute of Immunology), CLIA (Architect HBc II; Abbott), and CLEIA (Lumipulse; Fujirebio), with cut-off points of 2^5, 1.0 (sample per cut-off [S/CO]), and 1.0 (COI), respectively. Anti-HBs was detected using PHA (Mycell II anti-HBs; Institute of Immunology), CLIA (Architect Osabu; Abbott), and CLEIA (Lumipulse; Fujirebio) with cut-off points of 2^3, 10.0 mIU/mL, and 10 mIU, respectively. Hepatitis B envelope antigen (HBeAg) and anti-HBe were tested by immunochromatography using laboratory reagents. We considered the presence of HBsAg and/or anti-HBc seropositivity as indicative of HBV exposure. Cases positive for anti-HBs and negative for both HBsAg and anti-HBs were designated as "estimated serological vaccination". Hepatitis B virus DNA was evaluated by real-time PCR and nested PCR, using primers derived from HBV surface genes. Nucleic acids were extracted from each HBsAg-positive serum sample and from the pooled sera of every 20 HBsAg-negative samples.

To investigate HCV infection, anti-HCV was detected using particle agglutination (PA) (Ortho HCV Ab PA test II; Ortho-Clinical Diagnostics, Tokyo, Japan), chemiluminescence immunoassay (Architect anti-HCV; Abbott), and CLEIA (Lumipulse II Ortho HCV; Ortho-Clinical Diagnostics), with cut-off points of 2^4, 1.0 (S/CO), and 1.0 (COI), respectively. Hepatitis C virus RNA was detected using real-time polymerase chain reaction (PCR) and nested reverse transcription–PCR with primers deduced from conserved regions in the 5′-non-coding region. Nucleic acids were extracted from the serum of each anti-

Table 1 Hepatitis B virus-specific oligonucleotide primers used in the current study

(a) Primers used in polymerase chain reactions (PCR)

	Stage-polarity	Primer name	Nucleotide position	Nucleotide sequence (5′–3′)
Primer set A	PCR 1st sense	WA-L	1862–1885	ACTGTTCAAGGGTCCAAGCTGTGC
	PCR 1st antisense	WA-R	1806–1829	AGCAAAAAGTTGCATGGTGCTGGT
	PCR 2nd sense	WA-2 L	1887–1908	GGTGGCTTTRGGRCATGGACAT
	PCR 2nd antisense	WA-2R	1781–1802	CAGACCAATTTATGCCTACAGC
Primer set B	PCR 1st sense	S1	1414–1434	ACGTCCTTTGTTTACGTCCCG
	PCR 1st sense	S2	1436–1456	CGGCGCTGAATCCCGCGGACG
	PCR 1st antisense	AS1	2130–2110	TCCAAATTACTTCCCACCCAG
	PCR 1st antisense	AS2	2160–2140	CTGACTACTAATTCCCTGGAT
	PCR 2nd sense	S3	1489–1508	CCGCTTCTCCGTCTGCCGTA
	PCR 2nd sense	S4w	1527–1547	CACCTCTCTTTACGCGGWCTC
	PCR 2nd antisense	AS3	2185–2165	TAGGCCCATATTAACATTGAC
	PCR 2nd antisense	AS4	2098–2078	CA TCAACTCACCCCAACACAG

(b) Primers used in full-length sequence

	Primer name	Nucleotide position	Nucleotide sequence (5′–3′)
Primer set A	WA-2 L	1887–1908	GGTGGCTTTRGGRCATGGACAT
	WA-2R	1781–1802	CAGACCAATTTATGCCTACAGC
	FA2R	217–240	GGTATTGTGAGGADDYTTGTCAAC
	FA4L	801–820	GTATTGGGGGCCAAGTCTGT
	FA4LAS	801–820	ACAGACTTGGCCCCCAATAC
	FA3L	107–124	CTGCTGGTGGCTCCAGTT
	P4AS	455–474	CAAGGTTATGTTGCCCGTTTG
	B260	260–279	AGAAAA TTGAGAGAAGTCCA
	B1260	1260–1279	GCCGATCCATACTGCGGAAC
	B2466	2466–2485	GTAAAGTTTCCCACCTTATG
	B2830	2830–2849	ATGCTGTAGCTCTTGTTCCC
Primer set B	S4w	1527–1547	CACCTCTCTTTACGCGGWCTC
	AS4	2098–2078	CA TCAACTCACCCCAACACAG

Table 2 Sex- and birth year-specific prevalence of hepatitis B surface antigen (HBsAg), anti-hepatitis B core (anti-HBc), anti-HBs, and anti-hepatitis C virus (anti-HCV) and "estimated serological vaccination" rates among elementary school students in Siem Reap, Cambodia

		n	HBsAg positive				Anti-HBc positive				Anti-HBs positive		
		(M, F)	*n*	(%)	(95% CI)	*P*-value†	*n*	(%)	(95% CI)	*P*-value†	*n*	(%)	(95% CI)
Total		248	5	(2.02)	(0.27–3.77)		27	(10.89)	(7.01–14.76)		40	(16.13)	(11.55–20.71)
Sex	Male	107	3	(2.80)	(0.00–5.93)	0.7545	14	(13.08)	(6.69–19.47)	0.4462	14	(13.08)	(6.69–19.47)
	Female	141	2	(1.42)	(0.00–3.37)		13	(9.22)	(4.44–14.00)		26	(18.44)	(12.04–24.84)
Birth year	1999–2000	15 (4, 11)	1	(6.67)	(0.00–19.29)	0.3528	1	(6.67)	(0.00–19.29)	0.0595	1	(6.67)	(0.00–19.29)
	2001	33 (12, 21)	0	(0.00)	(0.00–11.18)‡		6	(18.18)	(5.02–31.34)		6	(18.18)	(5.02–31.34)
	2002	56 (28, 28)	2	(3.57)	(0.00–8.43)		9	(16.07)	(6.45–25.69)		8	(14.29)	(5.12–23.45)
	2003	64 (31,33)	1	(1.56)	(0.00–4.60)		7	(10.94)	(3.29–18.58)		10	(15.63)	(5.55–22.58)
	2004	49 (23, 26)	1	(2.04)	(0.00–6.00)		3	(6.12)	(0.00–12.84)		7	(14.29)	(4.49–24.08)
	2005	31 (9, 22)	0	(0.00)	(0–11.90)‡		1	(3.23)	(0.00–9.45)		8	(25.81)	(10.40–41.21)

"Estimated serological vaccination" means positive for anti-HBs and negative for both HBsAg and anti-HBc.
†χ^2-Test or Cochran–Armitage trend test.
‡Poisson distribution.
*Statistically significant.
CI, confidence interval.

HCV positive sample and from the pooled sera of every 20 anti-HCV negative samples. We considered the presence of HCV RNA and/or anti-HCV seropositivity as indicative of HCV exposure.

Hepatitis B virus full-length genome sequencing

Hepatitis B virus DNA was amplified from all HBV DNA detectable cases by PCR with two sets of partially overlapping primers (set A and B) (Table 1a). Polymerase chain reaction products were generated using PrimeSTAR GXL (Takara Bio Inc., Shiga, Japan) in both first- and second-round PCRs. A 3730xl DNA sequencer and BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) were used for full-length sequencing. We used the sequence primer sets A and B, as shown in Table 1(b).

Statistical analysis

The χ^2-test was used to evaluate differences in the prevalence rates of HBsAg, anti-HBc, anti-HBs, HBV-DNA, anti-HCV, and HCV-RNA by sex. The Cochran–Armitage trend test was used to calculate trends associated with birth years. A multivariate logistic regression analysis with a stepwise selection method was carried out to identify the potential risk factors related to HBV and HCV infection. A *P*-value <0.05 was considered statistically significant.

Incidence survey

Students who participated in the survey more than twice and initially tested negative for HBsAg, anti-HBc, and anti-HBs were included in an HBV incidence survey (Fig. 1). Students with an estimated serological vaccination after entry were excluded from this survey. New HBV infections were diagnosed in students who became positive for HBsAg and/or anti-HBc.

Students who participated in the survey more than twice and initially tested negative for HCV-RNA and anti-HCV were included in an HCV incidence survey. New HCV infections were diagnosed in students who seroconverted to anti-HCV, with or without HCV-RNA.

Ethical issues

This study was approved by the ethics committee for epidemiological research of Hiroshima University (ethical no. 370–1) and by the Ministry of Health, Cambodia (ethical no. 0085 NECHR). We also obtained approval from the Ministry of Health, Cambodia (No.1494 NPH) for the transportation of Cambodian serum to Japan.

Table 2 Sex- and birth year-specific prevalence of hepatitis B surface antigen (HBsAg), anti-hepatitis B core (anti-HBc), anti-HBs, and anti-hepatitis C virus (anti-HCV) and "estimated serological vaccination" rates among elementary school students in Siem Reap, Cambodia

	Anti-HBs positive	Estimated serological vaccination				Anti-HCV positive				HCV-RNA positive			
	P-value†	*n*	(%)	(95% CI)	*P*-value†	*n*	(%)	(95% CI)	*P*-value†	*n*	(%)	(95% CI)	*P*-value†
Total		25	(10.08)	(6.33–13.83)		7	(2.82)	(0.76–4.88)		0	0	(0.00–1.49)‡	
Sex	0.4155	7	(6.54)	(1.86–11.23)	0.2157	2	(1.87)	(0–4.44)	0.6872	0	0	(0.00–3.45)‡	1.0
		18	(12.77)	(7.26–18.27)		5	(3.54)	(0.49–6.60)		0	0	(0.00–2.62)‡	
Birth year	0.2640	1	(6.67)	(0.00–19.29)	0.0269*	0	(0.00)	(0.00–100.00)*	0.0640	0	0	(0.00–100.00)‡	1.0
		2	(6.06)	(0.00–14.20)		2	(6.06)	(0.00–14.20)		0	0	(0.00–11.18)‡	
		4	(7.14)	(0.40–13.89)		3	(5.36)	(0.00–11.25)		0	0	(0.00–6.59)‡	
		5	(7.81)	(1.23–14.39)		1	(1.56)	(0.00–4.60)		0	0	(0.00–5.76)‡	
		6	(12.24)	(3.07–21.42)		1	(2.04)	(0.00–6.00)		0	0	(0.00–4.67)‡	
		7	(22.58)	(7.86–37.30)		0	(0.00)	(0.00–11.90)‡		0	0	(0.00–11.90)‡	

RESULTS

FROM ACADEMIC YEAR 2011 to 2013, 234 students (81.8%) of a total 286 3rd grade students participated in this study. In academic years 2014–2015, 135 students (44.9%) out of a total 301 5th and 6th grade students participated again as the same cohort for the incidence study. Therefore, a total of 248 students with 369 samples were enrolled in this survey. The subjects comprised 107 boys and 141 girls, with a mean age of 9.19 ± 1.12 years (range, 7–14 years) among 3rd-graders and 11.87 ± 1.11 years (range, 10–15 years) among 5th- and 6th-graders.

Prevalence survey

Prevalence of HBV infection

The overall HBsAg prevalence was 2.02% (5/248; 95% confidence interval [CI], 0.27–3.77), with rates of 2.80% (3/107; 95% CI, 0–5.93) and 1.42% (2/141; 95% CI, 0–3.37) among male and female students, respectively. Anti-HBc was detected in all HBsAg-positive cases. The anti-HBc and anti-HBs positivity rates were 10.89% (27/248; 95% CI, 7.01–14.76) and 16.13% (40/248; 95% CI, 11.55–20.71), respectively. The estimated serological vaccination rate was 10.08% (25/248; 95% CI, 6.33–13.83) (Table 2).

There were no significant differences between sexes in the prevalence rates of HBsAg, anti-HBc, and anti-HBs or the rate of estimated serological vaccination. Furthermore, the questionnaire responses did not reveal any factors related to the prevalence of HBsAg or anti-HBc (Table 3).

Hepatitis B surface antigen-positive cases

Hepatitis B virus DNA was detected in all five cases of HBsAg-positive students, all of which revealed genotype C (Table 4). In addition, HBeAg was detected in three cases (two boys, one girl), and anti-HBe was detected in two cases (one boy, one girl). The titer of HBV-DNA viral load was detected three times per sample in all five students. The result of HBV DNA viral loads was within the range of 1.0×10^2 copies/mL for Case 1, 3.00×10^6 copies/mL for Case 2, 3.2×10^7 copies/mL for Case 3, 1.0×10^8 – 7.1×10^9 copies/mL for Case 4, and 1.0×10^7 – 1.3×10^8 for Case 5. The full-length genome sequence for the HBV isolates were obtained from samples in four cases except Case 1 (Fig. 2). All of these samples were classified as subgenotype C1. Case 4 (female; born 2003) had three samples (N12–0092, N13–0095, and N14–0157) over 2 years, and case 5 (male; born 2004) had three samples (N12–0133, N14–0177, and N15–0051) over 3 years. There were no mutations in any case within the observation periods. Case 3 (female; born 2002; N14–0202) and case 4 shared 100% identity. Case 2 (male; born 2002; N11–0261) and case 5 also shared high sequence homology (99.1%). Cases 3 and 2 were 98.7% and Case 5 was 98.1% genetically identical to Case 3 and Case 4, respectively (Table 5).

Table 3 Univariate and multivariate analyses of risk factors for hepatitis B virus (HBV) and hepatitis C virus (HCV) infection among elementary school students in Siem Reap, Cambodia

	n	HBsAg						Anti-HBc						Anti-HCV					
		Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis			Univariate analysis			Multivariate analysis		
		OR	(95% CI)	*P*-value	AOR	(95% CI)	*P*-value	OR	(95% CI)	*P*-value	AOR	(95% CI)	*P*-value	OR	(95% CI)	*P*-value	AOR	(95% CI)	*P*-value
Sex																			
Male	107	2	(0.3–12.2)	0.442	–			1.5	(0.7–3.3)	0.3332	–			0.5	(0.1–2.7)	0.4297	–		
Female	141	1						1						1					
Birth year																			
1999–2000	15	4.1	(0.4–39.1)	0.1861				0.6	(0.1–4.5)	0.5882	1			0	–†	0.4959			
2001	33	0	–†	0.3762	–			2.1	(0.8–5.5)	0.1485	3.3	(1.04–9.7)	0.0426*	0	–†	0.293	–		
2002	56	2.3	(0.4–14.3)	0.3466	–			1.9	(0.8–4.4)	0.1569	2.7	(1.01–6.9)	0.0475*	0.6	(0.1-4.8)	0.5944	–		
2003	64	0.7	(0.1–6.5)	0.7644	–			1	(0.4–2.5)	0.988	–			1.2	(0.2–6.1)	0.8653	–		
2004	49	1	(0.1–9.3)	0.989	–			0.5	(0.1–1.6)	0.2319	–			1.65	(0.3–8.8)	0.5525	–		
2005	31	0	–†	0.3932	–			0.2	(0.03–1.8)	0.1432	–			2.9	(0.5–15.8)	0.1921	–		
Healthy																			
Yes	162	2.1	(0.2–19.1)	0.5003	–			0.8	(0.4–1.9)	0.6237	–			1.3	(0.2–6.9)	0.7523	–		
No	84	1						1						1					
Current periodic treatment																			
Yes	99	0.4	(0.04–3.3)	0.3474	–			0.7	(0.3–1.7)	0.4271	–			3.8	(0.7–20.0)	0.0897	4.1	(0.8–29.7)	0.0813
No	146	1						1						1			1		
HB vaccinated																			
Yes	80	3.2	(0.5–19.7)	0.1801	–			1.8	(0.8–4.1)	0.1513	2.2	(0.9–5.3)	0.0656	2.9	(0.6–13.3)	0.1531	3.6	(0.8–19.5)	0.1053
No	168	1						1			1			1			1		
History of operation																			
Yes	10	0	–†	0.6405	–			0.9	(0.1–7.3)	0.9127	–			0	–†	0.5789	–		
No	234	1						1						1					
History of blood transfusion																			
Yes	3	0	(0–135)	0.8006	–			0	–†	0.5378	–			0	–†	0.7641	–		
No	240	1						1						1					
Tattoo																			
Yes	1	0	–†	0.8843	–			0	–†	0.7225	–			0	–†	0.8627	–		
No	241	1						1						1					
Holes for pierced earrings																			
Yes	120	0.3	(0.03–2.3)	0.1842	0.3	(0.01–1.7)	0.1692	0.6	(0.2–1.3)	0.1735	–			2.6	(0.5–13.8)	0.2365	2.7	(0.6–19.8)	0.2202
No	123	1						1						1					
Logistic regression analysis with a stepwise selection method:					R^2 = 0.0388, model *P* = 0.1692, *n* = 243						R^2 = 0.0488, model *P* = 0.0396*, *n* = 248						R^2 = 0.1045, model *P* = 0.0848, *n* = 242		

†No positives.

*Statistically significant variables.

–, not selected by stepwise selection method. AOR, adjusted odds ratio; CI confidence interval; HBc, hepatitis B core; HBs, hepatitis B surface; HBsAg, hepatitis B surface antigen; OR odds, ratio.

Table 4 Hepatitis B surface antigen (HBsAg)-positive cases among elementary school students in Cambodia

Case	Birth year	Sex	Year of participation	HBsAg	Anti-HBc	Anti-HBs	HBeAg	Anti-HBe	HBV-DNA Quantification (copies/mL)	HBV genotype	Subgenotype	Answer to questionnaire: Healthy now	Periodic treatment	HB vaccinated	History of operation	History of blood transfusion	tattoo	Holes for pierced earrings
Case 1	2000	Male	2011†	11 +	12 +	—	—	+	+	1.0×10^2	C	Not detected	No	Yes	Yes	No	No	No	No
			2012‡	2000 +	275.2 +	1.6 -	—	+	+	1.0×10^2	C		Yes	No	Yes	No	No	No	No
Case 2	2002	Male	2011‡	2000 +	259.3 +	1.4	—	—	+	3.0×10^6	C	C1	Yes	No	No	No	No	No	No
Case 3	2002	Female	2014‡	2000 +	3.7 +	0.6	—	+	+	3.2×10^7	C	C1	Yes	No	No	No	No	No	No
Case 4	2003	Female	2012‡	2000 +	247.8 +	1.5	—	—	+	8.0×10^8	C	C1	Yes	No	Yes	No	No	No	Yes
			2013‡	2000 +	234.2 +	1.6	—	—	+	7.1×10^9	C	C1	No	No	N/A	No	No	No	Yes
			2014‡	2000 +	181 +	1.6	—	—	+	1.0×10^8	C	C1	Yes	No	No	No	No	No	Yes
Case 5	2004	Male	2012‡	2000 +	300 +	1.1	—	—	+	6.0×10^7	C	C1	Yes	No	Yes	No	No	No	no
			2014‡	2000 +	300 +	2.4	—	—	+	1.0×10^7	C	C1	Yes	No	No	No	No	No	no
			2015‡	2000 +	300 +	1.6	—	Not tested	+	1.3×10^8	C	C1	yes	No	Yes	No	No	No	No

†HBsAg by reversed passive hemagglutination; anti-hepatitis B core (HBc) and anti-hepatitis B surface (HBs) by passive hemagglutination.
‡HBsAg, anti-HBc, anti-HBs by chemiluminescent enzyme immunoassay.
HB, hepatitis B; HBeAb, hepatitis B envelope antibody; HBeAg, hepatitis B envelope antigen; N/A, No answer.

After investigating the strains registered with the International Nucleotide Sequence Databases Collaboration, including the DNA Data Bank of Japan, it was found that Case 2 shared 99.2% homology with the FJ023643 Laos strain and 98.6% with GQ924636 (Malaysia). Cases 3 and 4 shared 98.9% homology with GQ358154 (Indonesia) and 98.6% with FJ349225 (Belgium). Although the AB031265 strain (Vietnam) was classified as genotype C1, the homology rates were lower (96.1–96.6%) (Table 5).

Hepatitis B infection trends according to birth year

The prevalence rates of HBsAg and anti-HBs did not show significant trends by birth year ($P = 0.7422$ and $P = 0.4946$, respectively, by Cochran–Armitage trend test) (Fig. 3a–c). In contrast, the prevalence of anti-HBc decreased significantly according to birth year after 2001 ($P = 0.0135$) (Fig. 3b), whereas the rate of estimated serological vaccination increased significantly by birth year ($P = 0.0229$) (Fig. 3d).

Evaluation of HBV infection based on questionnaire responses

A total of 32.3% (80/248) students responded that they had received HB vaccinations. Among them, 7.5% (95% CI, 1.73–13.27; 6/80) were identified as having estimated serological vaccination, 15.0% (95% CI, 7.18–22.82; 12/80) were anti-HBc positive, and three were HBsAg positive.

One hundred and sixty-eight students (67.7%) reported that they did not receive the HB vaccination. Among 168 students, 19 (11.31%; 95% CI, 6.52–16.10) were identified as having estimated serological vaccination and 15 students (8.93%; 95% CI, 4.62–13.24) were anti-HBc positive.

Prevalence of HCV infection

The anti-HCV prevalence was 2.82% (7/248; 95% CI, 0.76–4.88). Hepatitis C virus RNA was undetectable (0/248; 95% CI, 0–1.49) in all anti-HCV positive and negative cases (Table 2).

Furthermore, no significant trend in HCV exposure was observed by birth year ($P = 0.0802$) (Fig. 3e), and no factors related to the prevalence of anti-HCV were identified from the questionnaire responses (Table 3).

Incidence survey

From 2011 to 2015, 96 of 248 students participated in the survey more than twice (Fig. 1).

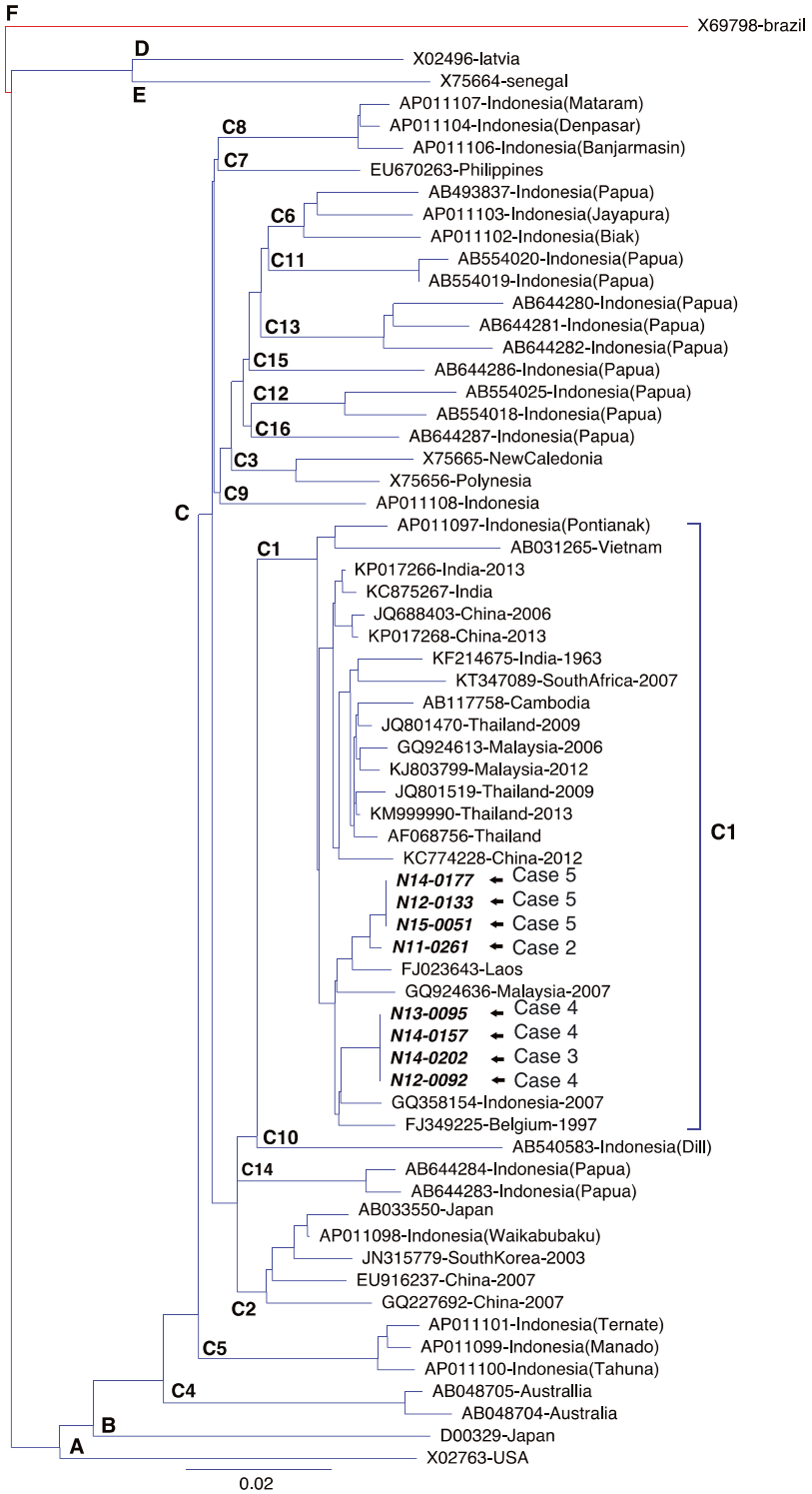

Figure 2 Phylogenetic tree constructed from full-length hepatitis B virus (HBV) genomes using the neighbor-joining method. Eight samples from four cases of HBV infection among elementary school students in Siem Reap, Cambodia, were classified as C1. There were no mutations in Case 4 (N12–0092, N13–0095, and N14–0157) or Case 5 (N12–0133, N14–0177, and N15–0051). Cases 3 and 4 shared 100% identity.

Table 5 Identity of the full hepatitis B virus (HBV)-DNA sequence among four hepatitis B surface antigen-positive elementary school students in Siem Reap, Cambodia

HBV genotype	Isolate	Identity, %							
		N12–0092 (Case 4)	N13–0095 (Case 4)	N14–0157 (Case 4)	N14–0202 (Case 3)	N11–0261 (Case 2)	N12–0133 (Case 5)	N14–0177 (Case 5)	N15–0051 (Case 5)
	N13–0095 (Case 4)	100.0							
	N14–0157 (Case 4)	100.0	100.0						
	N14–0202 (Case 3)	100.0	100.0	100.0					
	N11–0261 (Case 2)	98.7	98.7	98.7	98.7				
	N12–0133 (Case 5)	98.1	98.1	98.1	98.1	99.1			
	N14–0177 (Case 5)	98.1	98.1	98.1	98.1	99.1	100.0		
	N15–0051 (Case 5)	98.1	98.1	98.1	98.1	99.1	100.0	100.0	
C1	AB031265 – Vietnam	96.6	96.6	96.6	96.6	96.6	96.1	96.1	96.1
C1	JQ688403 – China	98.8	98.8	98.8	98.8	98.7	98.2	98.2	98.2
C1	AB117758 – Cambodia	97.8	97.8	97.8	97.8	97.7	97.2	97.2	97.2
C1	JQ801519 – Thailand	98.3	98.3	98.3	98.3	98.2	97.7	97.7	97.7
C1	FJ023643 – Laos	98.6	98.6	98.6	98.6	99.2	98.5	98.5	98.5
C1	GQ924636 – Malaysia	98.5	98.5	98.5	98.5	98.6	98.0	98.0	98.0
C1	GQ358154 – Indonesia	98.9	98.9	98.9	98.9	98.7	98.1	98.1	98.1
C1	FJ349225 – Belgium	98.6	98.6	98.6	98.6	98.5	97.9	97.9	97.9
C2	AB033550 – Japan	96.4	96.4	96.4	96.4	96.3	95.7	95.7	95.7
C3	X75656 – Polynesia	95.6	95.6	95.6	95.6	95.4	94.7	94.7	94.7
C4	AB048704 – Australia	93.2	93.2	93.2	93.2	93.1	92.4	92.4	92.4
A	X02763	91.4	91.4	91.4	91.4	91.3	90.8	90.8	90.8

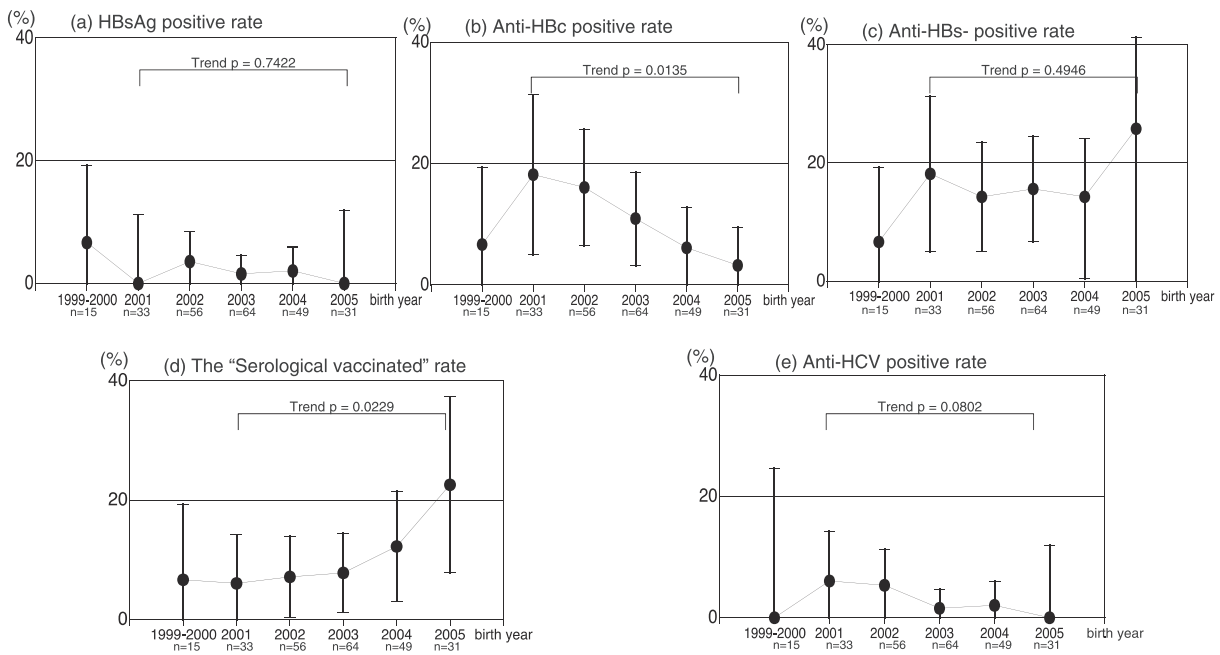

Figure 3 Birth year-specific rates of hepatitis B virus (HBV) and hepatitis C virus (HCV) infection and "estimated serological vaccination" rates among elementary school students in Siem Reap, Cambodia. (a) Prevalence of hepatitis B surface antigen (HBsAg). (b) Prevalence of anti-hepatitis B core (HBc). (c) Prevalence of anti-HBs. (d) Estimated serological vaccination rate (positive for anti-HBs and negative for both HBsAg and anti-HBc). (e) Prevalence of anti-HCV. Values are shown with 95% confidence intervals.

Incidence of HBV infection

Among the 96 eligible students, 72 students initially tested negative for HBsAg, anti-HBc, and anti-HBs. After excluding two students with estimated serological vaccination, 70 students were the subjects of the HBV incidence survey. During a 12–52-month period (179.33 person-years [PY]), HBsAg and/or anti-HBc seropositivity were absent in all cases. The incidence of HBV infection was calculated as 0/1000 PY (95% CI, 0–20.61/1000 PY).

Incidence of HCV infection

Among the 96 eligible students, 93 who initially were negative for HCV RNA and anti-HCV were included in the HCV incidence survey. During a 12–52-month period (254.3 PY), HCV-RNA remained undetectable in all cases. No case turned to positive for anti-HCV. The incidence of HCV infection was calculated as 0/1000 PY (95% CI, 0–14.50/1000 PY).

DISCUSSION

IN THIS STUDY, we investigated the prevalence and incidence of HBV and HCV infection among elementary school students in Siem Reap province, Cambodia who were born after the national infant HB vaccination program was established. This study was carried out at Sasar Sdam elementary school, which is located in a rural area. In Cambodia, the classification of urban and rural areas was established in 2004. An urban area classification is made according to three criteria: a population density $>200/km^2$, percentage of male employment in agriculture $<50\%$, and a total community population exceeding 2000; all other areas were defined as rural. In 2008, the rural population comprised 80.5% of 13.4 million in the total population.[10]

In 2001, the Cambodian government introduced a three-dose infant HB vaccination schedule (6, 10, and 14 weeks of age). In 2005, this schedule was replaced by a universal HB four-dose vaccination schedule that included a birth dose.[8,9] In a study undertaken in a moderately developed area, Soeung *et al.*[9] reported that the HBsAg positivity rate among 5-year-old children born in 2001, before the implementation of this vaccination program, was 2.9%. In 2011, Mao *et al.*[8] investigated the prevalence of HBsAg among children in a rural zone who were born in 2006 (after implementation of the HB vaccination program), and reported a rate of 1.41%. According to these reports, the decrease in positivity rates among 5-year-old children after HB vaccination implementation was limited to HBsAg levels.

In our survey of HBV infection, the prevalence rates of HBsAg, anti-HBc, and anti-HBs positivity were 2.02%, 10.89%, and 16.13%, respectively. In this first study carried out among Cambodian children, we investigated all three parameters to clarify the effects of HB vaccination and HBV exposure. The rate of estimated serological vaccination was found to significantly increase according to birth year, suggesting that increasing numbers of students acquired anti-HBs through vaccination after the national program was introduced.

Nearly 90% of cases involving vertical transmission from the mother who was infected with HBV persistently at birth result in persistent HBV infections. Regarding horizontal infection transmission, 80–90% of infants infected in their first year and 30–50% of children infected before the age of 6 years will develop chronic infections.[2] In this study, five HBsAg-positive cases were identified among the 27 anti-HBc-positive cases. The remaining 22 cases were considered acute infections, suggesting the occurrence of horizontal transmission or vertical transmission. Case 3, a female student born in 2002, and Case 4, a female student born in 2003, shared 100% homology in the HBV phylogenetic analysis. We confirmed that Cases 3 and 4 were not born to the same mother through a mitochondrial gene sequence analysis; therefore, the high identity of the HBV genome was suggested to be caused by horizontal transmission.

We did not detect new HBV infection cases in this study. The rarity of HBV transmission would be attributable to the low prevalence of HBsAg (2.02%) and improvements in children's hygiene, especially at home and at school. These results indicate that the rate of HBV exposure is decreasing significantly among students born after 2001, who therefore have lower risk of horizontal HBV transmission. Accordingly, the prevalence of HBsAg is expected to decrease below 1% in the near future.

Among the students who reported receiving HB vaccination, only 7.5% were identified as having estimated serological vaccination. However, 15.0% of these subjects showed HBV exposure. The possible reasons behind this discrepancy include the inadequate timing of HB vaccination, insufficient number of HB vaccination doses, inadequate vaccine quality, failure to acquire antibody seropositivity due to individual conditions, and recall errors. Furthermore, the questionnaire responses collected from guardians were recall-based, rather than from the vaccination records; accordingly, the responses may not have been accurate. Additionally, although the reported BD and three-dose HB vaccination coverage rate among rural Cambodian children born in 2006 was 82%,[8] this result was not supported by serological testing.

No cases of chronic HCV infection were detected in our study, and the anti-HCV prevalence was 2.82%. In addition, new HCV infection did not occur in this survey. Previous reports found that the prevalence of anti-HCV was 0% among healthy children[11] and 14.7% among adult blood donors.[5] Yamada *et al.*[4] reported an anti-HCV prevalence of 2.3%, as well as a significant trend over time for both anti-HCV and HCV-RNA positivity.

Vong *et al.*[12] reported that Cambodia has one of the highest overall treatment injection usage rates worldwide. Moreover, the needles and syringes are not managed safely, and many institutions practice inappropriate disposal methods. In addition to HB vaccination, basic safety and hygiene knowledge and practices must be enforced to prevent HBV and HCV transmission. According to Yamada *et al.*,[4] surgery and blood transfusion are potential risk factors for HBV and HCV infections, respectively, thus confirming the importance of improved medical safety and hygiene.

In conclusion, our study used HBV full-genome sequencing and serological analysis to suggest the possibility of horizontal transmission of HBV among elementary school students in Cambodia. Moreover, we observed a decreasing trend in the anti-HBc-positive rate with age, which corresponded to the increasing rate of estimated serological vaccination.

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