Table 5 4bOutput of logistic regression analyses (at registration) ($n = 1860$)

		HBsAg			Anti-HBc			Anti-HBs		
		OR	95% CI	*P*	OR	95% CI	*P*	OR	95% CI	*P*
Sex	*n*									
Female (Ref.)	752	1.00			1.00			1.00		
Male	1108	1.15	(0.59–2.29)	0.688	1.36	(1.07–1.72)	0.011	0.97	(0.73–1.29)	0.843
Age (years)	*n*									
≤49 (Ref.)	360	1.00			1.00			1.00		
50–69	1019	1.29	(0.61–3.06)	0.523	1.51	(1.10–2.09)	0.010	1.32	(0.91–1.96)	0.151
≥70	481	0.21	(0.03–0.86)	0.028	1.79	(1.25–2.59)	0.002	1.65	(1.07–2.58)	0.024
Introduced to dialysis	*n*									
Since 1991 (Ref.)	1372	1.00			1.00			1.00		
Before 1991	486	1.19	(0.58–2.32)	0.625	1.55	(1.20–2.00)	0.001	1.83	(1.35–2.46)	<0.001

CI, confidence interval; HBc, hepatitis B core; HBs, hepatitis B surface; HBsAg, hepatitis B surface antigen; OR, odds ratio; Ref., reference.

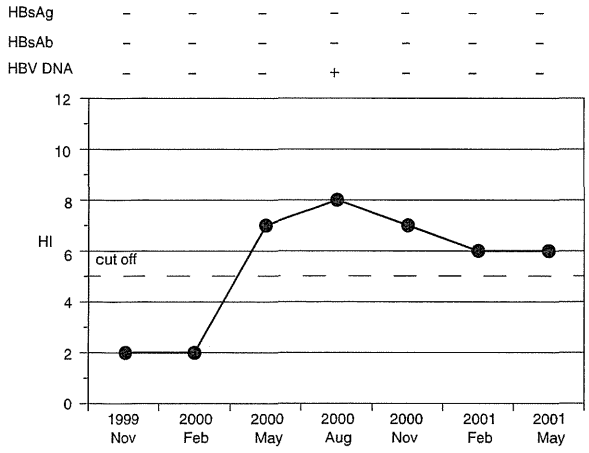

Figure 3 Changes of hepatitis B virus (HBV) markers in the those who converted to anti-hepatitis B core (HBc) positive status, implying new infection.

In this survey, the prevalence of HBsAg among 1860 patients including those who started HD from 1969 was 2.6%. But to evaluate the risk of HBV infection excluding the effect of blood transfusion, we investigated the 1372 patients who had started HD since 1991 when erythropoietin was approved by the health insurance policy in Japan, and the 486 patients before 1991. The prevalence of HBsAg among the 1372 patients who started HD since 1991 was 2.1%. On the other hand, the prevalence of HBsAg among the 486 patients who started before 1991 was 3.9%. The prevalence since 1991 was slightly lower than that before 1991, presumably due to the decreased risk of HBV infection from transfusion ($P = 0.048$) in univariate analysis and moreover by the logistic regression analysis; as for the risk of HBsAg, there is no significant difference in those who had started since 1991 and those before 1991.

The reported HBsAg positive rate was 2.4% according to a cross-sectional survey of 92 460 HD patients in 2006.[10] Another survey of 30 916 patients who underwent surgery in regional center hospitals in Hiroshima Prefecture between 1993 and 2001 reported that the prevalence of HBsAg was 1.8%.[11] Each of the studies, including this survey, showed higher prevalence of HBsAg among HD patients than that among first-time donors and the general population in Japan in medical investigations of hepatitis viruses (0.63%).[1] However, the prevalence of HBsAg at 2.1% among the 1372 patients who were introduced HD since 1991 is not much higher than that of the general population, particularly considering that Hiroshima Prefecture is an area with a high prevalence of HBsAg and the prevalence of HBsAg in first-time blood donors who were the same age as those in this study was 2.1% in the Chugoku district in 2000.[12]

Anti-HBc positive rate among 1372 patients who started HD since 1991 in this study indicated that the rate of exposure to HBV infection was 18.9%, but the anti-HBc positive rate among 486 patients who started before 1991 was 25.5%. Among the 486 patients, anti-HBc positive rate was higher than that among the patients who started HD treatment since 1991 ($P < 0.01$). In addition, by the logistic regression analysis of the risk of anti-HBc and anti-HBs, there is significant difference between "since 1991" and "before 1991". We considered that the risk of HBV infection is higher in the group who started HD before 1991 than since 1991.

The pilot survey was conducted in the same prefecture among 1637 company workers who had received health check-ups (mean age, 49.3 ± 14.9 years old; range, 19–81; unpublished data), and the anti-HBc positive rate was 18.0%, which is the same as among the HD patients obtained in this study.

This result showed that the risk of HBV infection among HD patients was not so significantly high and that HD patients may not produce anti-HBc sufficiently.[13]

Multivariate analysis indicated that the risk of being positive for HBsAg did not correlate with the duration of HD, but did correlate with age of 70 years or more. The younger generation has a significantly higher risk of persistent HBV infection ($P = 0.037$), but the older generation has high prevalence of anti-HBc (HBV exposure rate) and anti-HBs. Thus, these results show that the lower HBsAg positive rate with older age may be attributed to loss of HBsAg.

The prevalence of anti-HBs in female patients was slightly higher than that in male patients without statistical significance and that of HBsAg and anti-HBc in female patients was lower than male patients. We need to study spontaneous HBV clearance and the loss of HBsAg by sex.

The risk of anti-HBc positivity was twofold higher in patients aged 70 years or more. A significant risk factor for conversion to anti-HBs positivity was duration of HD of 5 years or more (range, 5–8). This finding suggests that exposure to HBV infection occurs in proportion to the length of time on HD.

In our study, the incidence rate of HBsAg was 0/1000 person-years (95% CI, 0–1.1/1000 person-years) and the incidence rate of HBV infection determined by conversion to anti-HBc positivity was 0.3/1000 person-years (95% CI, 0–1.6/1000 person-years). Thus, the incidence rate of HBV infection in HD patients would be slightly higher than that of first-time blood donors ($2.78/10^5$ person-years; 95% CI, $1.78–4.14/10^5$ person-years).[14] The observed incidence rate of HBV infection, determined by the conversion to HBsAg positivity and/or anti-HBc positivity, is much lower than the incidence rate of HCV infection in the same cohort of patients during the same observation period, at $330/10^5$ person-years. The difference may be attributed to the difference in carrier rates; while the HBV carrier rate in the group was 2.1% at the start of the survey, the HCV carrier rate is as high as 15.7% in the same cohort.[4] Such a wide difference between HCV and HBV could be due to the size of reservoir, which would be far larger for HCV than HBV in HD patients.[15] HBV infection resolves in most cases and rarely persists. Unlike a HCV carrier, a patient infected with HBV can seldom serve as a reservoir for further spread of HBV. We supposed that the discrepancy between incidence rate of HBV and that of HCV was caused by the difference of their carrier rates and of their characteristics for persistent infection. This fact could explain why the incidence of HBV is low. When designing and implementing preventive measures in a given HD center, it should be prerequisite to determine and take account of the size of the population of patients with persistent HBV and HCV.[16]

Hepatitis B virus DNA was examined to investigate occult HBV in HD patients who were negative for HBsAg, and two HBV DNA positive cases were detected. Neither case was in the early phase of infection. In one case, a blood sample was taken only once at registration, and further analysis could not be done because of their death. In the other case, anti-HBc was positive and HBsAg was negative, indicating the state of late phase of HBV infection or occult HBV status. HBsAg tests in general clinics show negative results in such cases because the quantity of antigen is below the limit of detection. These cases are thus regarded as "no existence of HBV" in the clinics. In this study, the prevalence of occult HBV of 0.11% (95% CI, 0.03–0.40%) was not so high.[17] We detected HBV DNA by real-time PCR in 20 samples via pooled NAT to find positive samples at first because we confirmed that we could detect over 10^1 copies/mL by real-time PCR and HBV DNA could be detected more than cut-off 10^2 copies/mL by pooled NAT. But there is a possibility that the prevalence may be underestimated by pooled NAT compared with individual NAT. In addition, serological tests were done using assay kits provided by the JRC, which is not more sensitive than other kits such as chemiluminescent immunoassay.

In this study, the prevalence of HBsAg and incidence rate of HBV infection were lower than usually expected in HD patients. Furthermore, the risk of new HBV infection did not increase during the course of HD. The HD centers participating in this study have been using a nosocomial infection prevention program to prevent HCV infection since 1999.[4] The low incidence rate of HBV infection observed in this study seems to be one of the effects of this program.

Western guidelines such as KDIGO (Kidney Disease: Improving Global Outcomes)[18] have established that HD patients should be vaccinated against HBV. However, it has been pointed out that HB vaccination in HD patients is not common in Japan despite this recommendation,[5] as observed in the subjects of this study. Changes in the HB vaccination policy in Japan may enable the rate of HBV infection in HD patients to approach zero.

To prevent new HBV infections in HD patients, it is prerequisite to grasp the size of HBV carriers in the group and make strategies for prevention.

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The Association of Hepatitis C Virus Infection With the Prognosis of Chronic Hemodialysis Patients: A Retrospective Study of 3,064 Patients Between 1999 and 2010

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The prevalence of hepatitis C virus (HCV) infection is high among patients receiving chronic hemodialysis. To clarify the risk ratio of HCV infection with respect to mortality and prognosis in chronic hemodialysis patients, a retrospective longitudinal cohort study was conducted in 2010 and involved 3,064 patients receiving chronic hemodialysis at nine dialysis facilities in Hiroshima, Japan, who were recruited from 1999 to 2003. Logistic regression and Cox hazards models were used to estimate the mortality risk among hemodialysis patients. Among the patients, 422 (14.0%) were positive for HCV RNA. HCV RNA positivity was associated with death in the logistic model (adjusted odds ratio = 1.79; $P < 0.001$). However, it was not a risk factor for the reduced of survival rate in the Cox proportional hazard model (adjusted risk ratio = 1.07; $P = 0.4138$). In summary, among hemodialysis patients, HCV RNA is correlated with the mortality rate; however, it is not significantly correlated with prognosis in terms of survival time. On the other hand, diabetes and age at dialysis onset are significantly correlated with survival. Diabetes control treatment should be preferentially selected for hemodialysis patients, and antiviral therapy for HCV should be introduced based on the clinical state of the patient. ***J. Med. Virol. 87:1558–1564, 2015.*** © 2015 The Authors. *Journal of Medical Virology* published by Wiley Periodicals, Inc.

KEY WORDS: hemodialysis; HCV; diabetes; prognosis

INTRODUCTION

According to the World Health Organization (WHO), approximately 3% of the global population has been exposed to the hepatitis C virus (HCV) and 130–150 million persons harbor persistent HCV infection, which has been strongly correlated with liver cancer. HCV is transmitted through exposure to contaminated blood. Hemodialysis patients are at a high risk of acquiring HCV infection because of the requirement for invasive treatment involving daily hemodialysis [Kumagai et al., 2005]. Although the number of new HCV infections in Japan is decreasing as a result of the Manual of Standard procedures for hemodialysis and nosocomial infection prevention, more than 90% of HCV infected hemodialysis patients develop chronic hepatitis. The HCV carrier rate remains higher among hemodialysis patients than among the general population. HCV carriers develop cirrhosis of the liver or hepatocellular carcinoma within 20–30 years of the initial infection [Kiyosawa et al., 1990; Seeff, 2002; Tanaka et al., 2003]. Hemodialysis patients reportedly have a high rate of death from cirrhosis of the liver or

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hepatocellular carcinoma [Espinosa et al., 2001]. On the other hand, HCV infection is associated with poor prognosis among hemodialysis patients [Fabrizi et al., 2007]. HCV infection control is very important because many patients on hemodialysis for a long time as a result of ongoing advances in medicine.

In Japan, additional public medical expenses for chronic viral hepatitis patients were initiated in 2008 and antivirus therapy is recommended. The Japanese Society for Dialysis Therapy generated clinical guidelines for the treatment of HCV infection. These guidelines stipulate that antivirus therapy should only be recommended to patients who are expected to have a good prognosis. The difference between the prognoses of hemodialysis patients with or without HCV infection and the risk ratio of HCV infection with respect to the mortality rate of hemodialysis patients were clarified, and thereby the usefulness of aggressive HCV infection management in these patients was discussed.

MATERIALS AND METHODS

Subjects

The subjects of this study were 3,096 hemodialysis patients attending nine dialysis facilities in Hiroshima, Japan from November 1999 to February 2003 who were also the subjects in our 2005 cohort study [Kumagai et al., 2005]. After excluding 32 patients (temporary dialysis: 2, missing data on the outcome: 3, missing data regarding the date of outcome: 23, missing data regarding the date of hemodialysis initiation: 3, missing data regarding HCV infection: 1), 3,064 patients were investigated in this study in October 2010 (Fig. 1). This study was conducted according to the Ethical Guidelines for Epidemiology Research in Japan and was approved by the Ethics Committee of Hiroshima University (Hiroshima Univ. Epi-294).

Methods

The patient outcomes and prognoses were surveyed from the medical records of nine dialysis facilities; the date and cause of death, date of hospital changes, cause of hemodialysis, the presence or absence of diabetes, and HBs antigen (HBsAg) and HCV RNA positivity were obtained, and all data were supplied anonymously. In our 2005 study, HCV RNA positivity was determined via polymerase chain reaction (PCR) with nested primers derived from well-conserved areas within the 5′-noncoding region of the HCV genome [Okamoto et al., 1990]. HBsAg positivity was determined via passive hemagglutination (PHA) with currently available commercial kits (Mycell anti-HBs, Institute of immunology Co. Ltd., Tokyo, Japan).

Statistical Analysis

The χ^2 test was used to compare frequencies between HCV infected and uninfected patients. The log-rank test was used to compare the vital prognosis of each group according to (a) HBsAg, (b) HCV RNA, (c) diabetes, (d) cause of hemodialysis, (e) year of birth, and (f) age at onset of hemodialysis. The logistic regression model and the Cox proportional hazards model were used to estimate the mortality risk. *P*-values <0.05 were considered statistically significant. Statistical analyses were performed with a software package SPSS (version 12.0; SPSS, Inc., Chicago, IL).

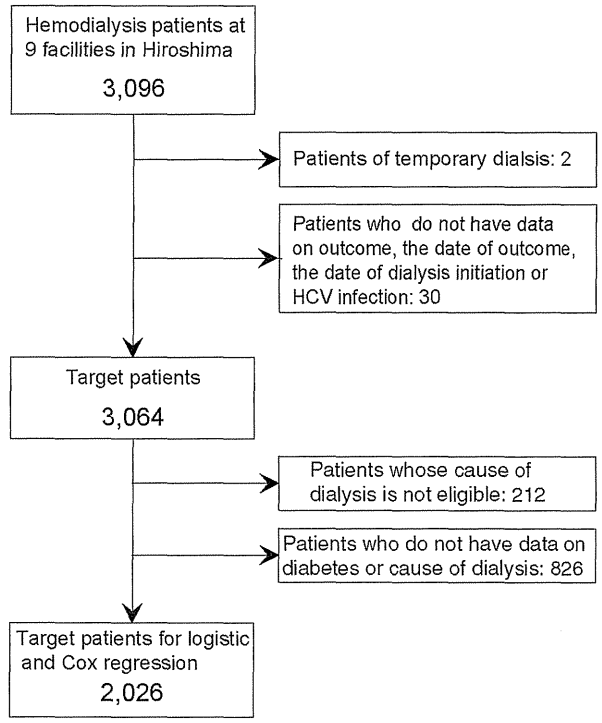

Fig. 1. Study subject. Target patients and target patients for logistic and Cox proportional hazard regression were selected as the flowchart.

RESULTS

Characteristics of Hemodialysis Patients

The subjects' background information is shown in Table I. Among the 3,064 total subjects, 1,802 (58.8%) were men and 1,262 (41.2%) were women. The average age at hemodialysis onset was 58.0 ± 15.8 years. The average duration of hemodialysis was 10.2 ± 7.5 years. Chronic glomerulonephritis was the most common cause of hemodialysis initiation (36.2%, 1,109 cases), followed by diabetic nephropathy (25.0%, 765 cases). Among the 212 cases (6.9%), the number of patients with polycystic kidney disease was highest with 70 cases (2.3%).

In addition, 947 cases, accounting for 30.9% of the total subjects, had concomitant diabetes. Additionally, 428 (14.0%) patients were HCV RNA positive and 73

TABLE I. Characteristics of study participants

	All patients	HCV RNA positive	HCV RNA negative	*P*-value
Total	3,064	428	2,636	
Gender				0.0003***
Men	1,802 (58.8%)	286 (66.8%)	1,516 (57.5%)	
Women	1,262 (41.2%)	142 (33.2%)	1,120 (42.5%)	
Year of birth				<0.0001***
1905–24	521 (17.0%)	45 (10.5%)	476 (18.1%)	
1925–44	1,597 (52.1%)	261 (61.0%)	1,136 (50.7%)	
1945 and above	946 (30.9%)	122 (28.5%)	824 (31.3%)	
Age at onset of hemodialysis				0.0005***
49 years or younger	904 (29.5%)	145 (33.9%)	759 (28.8%)	
50–59 years	634 (20.7%)	89 (20.8%)	545 (20.7%)	
60–69 years	723 (23.6%)	116 (27.1%)	607 (23.0%)	
70 years or older	803 (26.2%)	78 (18.2%)	725 (27.5%)	
Duration of hemodialysis				<0.0001***
5 years and below	815 (26.6%)	95 (22.2%)	720 (27.3%)	
5–10 years	971 (31.7%)	131 (30.6%)	840 (31.9%)	
10–15 years	623 (20.3%)	69 (16.1%)	554 (21.0%)	
15 years and above	655 (21.4%)	133 (31.1%)	522 (19.8%)	
Cause of hemodialysis initiation				<0.001***
Chronic glomerulonephritis	1,109 (36.2%)	147 (34.3%)	962 (36.5%)	
Diabetic nephropathy	765 (25.0%)	146 (34.1%)	619 (23.5%)	
Renal sclerosis	156 (5.1%)	16 (3.7%)	140 (5.3%)	
Other	212 (6.9%)	21 (4.9%)	191 (7.2%)	
Unknown	822 (26.8%)	98 (22.9%)	724 (27.5%)	
Diabetes				<0.0001***
No	1,908 (62.3%)	230 (53.7%)	1,678 (63.7%)	
Yes	947 (30.9%)	173 (40.4%)	774 (29.4%)	
Unknown	209 (6.8%)	25 (5.8%)	184 (7.0%)	
HBsAg				
Negative	2,991 (97.6%)	420 (98.1%)	2,571 (97.5%)	0.5620
Positive	73 (2.4%)	8 (1.9%)	65 (2.5%)	
HCV RNA				
Negative	2,636 (86.0%)			
Positive	428 (14.0%)			

Data are expressed as numbers (%). ***Parameters have significant difference ($P < 0.001$) between patients who were positive for HCV RNA or negative for HCV RNA.

(2.4%) patients were HBsAg positive; 8 (0.3%) patients were double positive.

Gender, year of birth, age at hemodialysis onset, duration of hemodialysis, cause of hemodialysis initiation, and diabetes all correlated significantly with HCV RNA positivity ($P < 0.001$); on the other hand, no significant association was observed between HBsAg and HCV RNA positivity ($P = 0.562$).

Outcome

Up to December 2010, 1,501 of 3,064 patients (49.0%) had died (Fig. 2). Death occurred significantly more frequently in HCV RNA positive patients (60.0%) than in HCV RNA negative patients (47.2%, $P < 0.001$).

The causes of death were similar to those in a 2010 reports from the Japanese Society for Dialysis

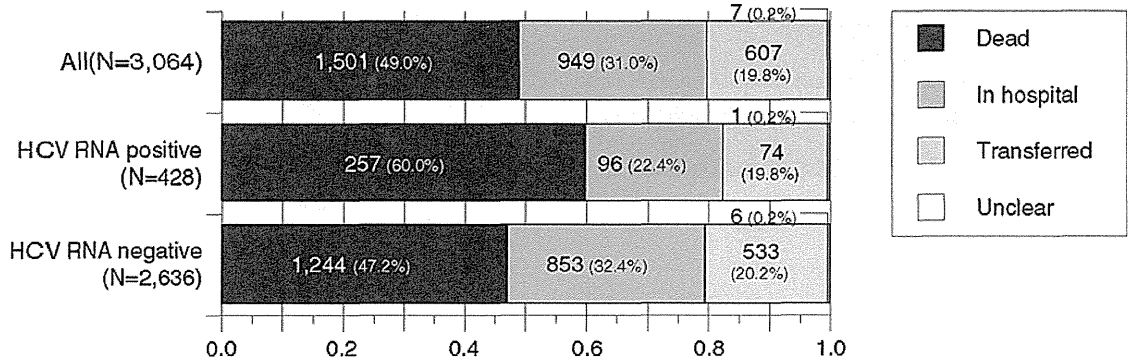

Fig. 2. Outcome according to infection status for HCV. Outcomes by all patients, HCV RNA positive patients, and HCV RNA negative patients are shown.

Therapy, the most common cause was cardiovascular diseases (430 cases, 29.2%) such as heart failure and myocardial infarction, followed by infectious disease (222 cases, 15.1%) (Fig. 3) and these causes of death were not related to HCV RNA positivity. Nevertheless the proportion of deaths from hepatic failure and HCC was higher among HCV RNA positive patients than among HCV RNA negative patients (8.7% vs. 1.6%).

Mortality and Prognosis

From the results of the logistic regression analysis shown in Table II, male gender ($P = 0.008$), year of birth between 1905 and 1944 ($P < 0.001$), age of 49 years or younger at hemodialysis initiation ($P = 0.021$), duration of hemodialysis exceeding 5 years ($P < 0.001$), diabetes ($P = 0.031$), and HCV RNA positivity ($P < 0.001$) were related to mortality. Regarding HBV infection, no significant correlation with mortality was found. On the contrary, the mortality risk was significantly lower among subjects with an age at dialysis onset of 70 years or older than among those aged 50–59 years and the cause of hemodialysis was not correlated with the mortality risk.

Regarding the survival duration, a univariate analysis conducted using the Kaplan–Meier method and log-rank test found that HCV RNA negative patients did not have a better prognosis than HCV RNA positive patients ($P = 0.646$; Fig. 4). Patients without diabetes ($P < 0.001$), with a more recent year of birth (1945–1986; $P < 0.001$), cause of hemodialysis (chronic glomerulonephritis; $P < 0.001$), and age at dialysis onset (younger than 49 years; $P < 0.001$) had a significantly better prognosis.

Additionally, the factors associated with prognosis in a multivariate analysis of survival duration were examined using a Cox proportional hazards model (Table II). Male gender ($P = 0.001$), age of 60 years or older at dialysis onset ($P < 0.001$), and diabetes ($P < 0.001$) were identified as the risk factors for prognosis related to an outcome of death. Regarding HCV infection, no significant relationship with prognosis was identified ($P = 0.414$).

DISCUSSION

In this study, the HCV RNA positivity rate among hemodialysis patients in Hiroshima was 14.0%, similar to the estimated prevalence of HCV among hemodialysis patients in Hiroshima in 2005 (12.9%) [Kumagai et al., 2005] and much higher than that in the overall Japanese population. The HCV carrier rates in the overall Japanese population were calculated to be 0.95% in 1995–2000 and 0.44% in 2001–2006 [Tanaka et al., 2004, 2011] and were estimated based on a database of first-time blood donors. Moreover, the reported incidence of HCV among hemodialysis patients in 2005 [Kumagai et al., 2005] was $330/10^5$ person-years, and this number was also higher than that among blood donors ($1.86/10^5$ person-years [Tanaka et al., 2008]). Although the Japanese Society for Dialysis Therapy developed a guideline for the prevention of HCV for hemodialysis patients, hemodialysis patients are considered a high risk group for HCV infection. Outside of Japan, a high risk of HCV infection has been observed among hemodialysis patients [Vallet-Pichard and Pol, 2013].

In Japan, a reported 38,055 patients were newly introduced to hemodialysis in 2012 [Nakai et al., 2014]. According to a report from the Japanese Society for Dialysis Therapy, the number of deaths among hemodialysis patients was 30,710 in 2012; therefore, the calculated cumulative number of hemodialysis patients in 2012 was 310,007. As Japan is a developed country, the number of patients with lifestyle related diseases, including those requiring

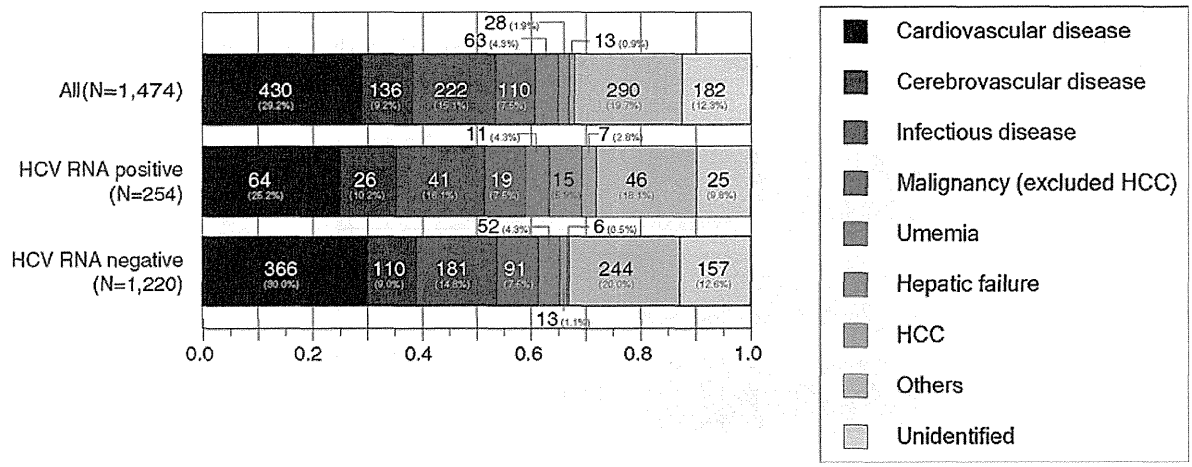

Fig. 3. Cause of death according to infection status for HCV. Among dead patients (N = 1,474), cause of death by all patients, HCV RNA positive patients, and HCV RNA negative patients are shown.

TABLE II. Results of Logistic and Cox Regression Analysis for Factors of Death (N = 2,238)

	Logistic regression for mortality[a]			Cox regression for prognosis[b]		
	AOR	95%CI	*P*-value	ARR	95%CI	*P*-value
Gender						
Men	1.30	(1.07–1.57)	0.0077**	1.19	(1.05–1.34)	0.0012**
Women	1.00			1.00		
Year of birth						
1905–24	13.91	(8.58–22.71)	<0.0001***	1.04	(0.77–1.41)	0.7836
1925–44	4.78	(3.47–6.60)	<0.0001***	0.99	(0.78–1.25)	0.9150
1945 and above	1.00			1.00		
Age at onset of hemodialysis						
49 years or younger	1.48	(1.06–2.06)	0.0208*	0.29	(0.22–0.37)	<0.001***
50–59 years	1.00			1.00		
60–69 years	0.81	(0.60–1.09)	0.1668	2.08	(1.72–2.52)	<0.001***
70 years or older	0.67	(0.46–0.99)	0.0417*	4.38	(3.47–5.49)	<0.001***
Duration of hemodialysis						
5 years and below	1.00					
5–10 years	0.43	(0.34–0.56)	<0.0001***			
10–15 years	0.28	(0.21–0.38)	<0.0001***			
15 years and above	0.22	(0.15–0.31)	<0.0001***			
Cause of hemodialysis initiation						
Chronic glomerulonephritis	1.06	(0.58–1.94)	0.8584	0.93	(0.65–1.30)	0.6766
Diabetic nephropathy	1.00			1.00		
Renal sclerosis	1.08	(0.60–1.97)	0.8085	1.39	(0.99–1.92)	0.0593
Other	1.28	(0.67–2.49)	0.4562	1.23	(0.82–1.81)	0.3008
Diabetes						
No	1.00			1.00		
Yes	1.88	(1.06–3.38)	0.0314*	1.96	(1.39–2.69)	0.0002***
HBsAg						
Negative	1.00			1.00		
Positive	1.04	(0.57–1.89)	0.8870	0.88	(0.57–1.29)	0.5277
HCV RNA						
Negative	1.00			1.00		
Positive	1.79	(1.37–2.35)	<0.0001***	1.07	(0.91–1.25)	0.4138

[a]$R^2 = 0.16$, model $P < 0.0001$, N = 2,238; AOR, adjusted odds ratio.
[b]Model $P < 0.0001$, N = 2,238; ARR, adjusted risk ratio.

hemodialysis, is increasing. The number of HCV carriers among hemodialysis patients possibly remains large. This study was conducted because the clarification of disease progression in and treatment decisions for hemodialysis patients with or without HCV infection are important topics.

The reported ALT levels in hemodialysis patients with or without HCV infection are generally low, reflecting a potentially severe level of liver injury. As a result of immunodeficiency, the natural course of HCV infection among hemodialysis patients differs from that among HCV infected patients not receiving hemodialysis. Therefore, even HCV infected hemodialysis patients with low ALT levels should not be considered healthy carriers, and as the current hemodialysis control has improved and the lifespans of hemodialysis patients are consequently longer, the Japanese Society for Dialysis Therapy has recommended that HCV infected hemodialysis patients in good condition should be treated with antiviral therapies.

The high mortality of HCV carriers receiving hemodialysis was confirmed by previous medical research reports and meta-analysis [Fabrizi et al., 2007, 2010].

In Japan, a cohort study involving nearly all hemodialysis patients in Iwate [Ohsawa et al., 2011] reported that the significant risk factors associated with prognosis were age, low serum albumin level, high CRP level, complication of diabetes, and HCV antibody positivity ($P = 0.043$). However, HCV antibody positivity was not a risk according to same analysis after excluding high-risk patients with additional complications such as cardiac infarction, cerebral embolism, or malignant neoplasm ($P = 0.106$). On the other hand, according to the results of a logistic regression to analyze the deaths of patients within 1 year based on a survey of 3,360 hospitals conducted by the Japanese Society for Dialysis Therapy, HBsAg positivity was not correlated with death, but HCV antibody positivity was correlated with a risk of death (the relative risk of a positive outcome for anti-HCV positivity was 1.2–1.4). In this study, HCV infection was defined as HCV RNA positivity whereas the Japanese Society for Dialysis Therapy defined infection as HCV positivity; additionally, the study interval was defined as the entire follow-up after certain duration whereas the Japanese Society for Dialysis Therapy defined the interval 1 year after hemodialysis initiation. Despite the different study

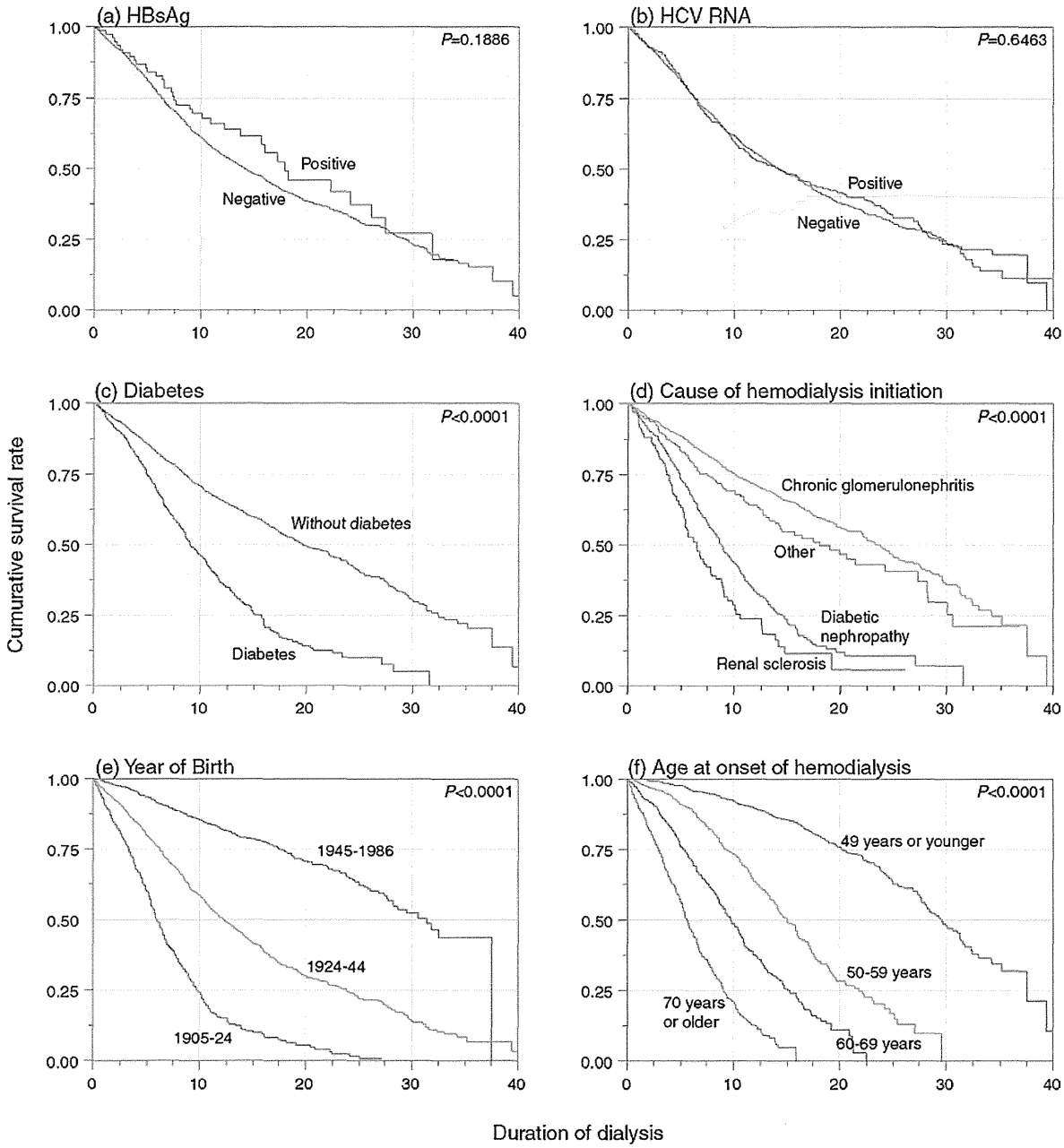

Fig. 4. Kaplan–Meier survival curve of mortality in hemodialysis patients. Survival curves of each groups classified by (**a**) HBs antigen, (**b**) HCV RNA, (**c**) diabetes, (**d**) cause of introduction of HD, (**e**) year of birth, (**f**) age at HD onset.

designs, both the logistic analysis and Cox's proportional hazard model results are in accordance with the results obtained in Iwate and by the Japanese Society for Dialysis Therapy.

We applied a logistic regression analysis to investigate the risk factors for death in hemodialysis patients and have confirmed that persistent HCV infection poses a significant risk of death after adjusting for HBsAg, Diabetes, Cause of hemodialysis, Year of birth, and Age at onset of dialysis as in previous studies.

However, a logistic regression is a type of cross-sectional analysis that considers the risk factors for death within a specified period without considering the observed survival duration of each subject. In this study, we attempted to analyze the same subjects while considering the observed survival duration of each subject.

The Cox's hazard regression analysis results show that the risk associated with the diabetes factor is greater than that associated with the persistent HCV infection factor. In this study, the reduction in survival time caused by diabetes is considered longer than that caused by HCV infection. This result seems to conflict with several reports in which HCV infection is a risk factor for death in hemodialysis patients. However, we do not deny that persistent HCV infection is a risk factor for death in hemodialysis patients.

However, we would like to demonstrate that although HCV infection is certainly a risk factor for death, diabetes has a stronger correlation with respect to survival duration. We would like to suggest another possible treatment strategy for HCV infected hemodialysis patients.

In this study, the main causes of death among dialysis patients were cardiac disorders and infectious disease, whereas liver disease only accounted for 2.8% of deaths (hepatic failure 1.9%, hepatocellular carcinoma 0.9%). In a survival analysis, the prognostic risk factor was diabetes rather than HCV infection. This finding suggests that diabetes care should be a priority for hemodialysis patients. HCV infection is certainly a risk factor for death among hemodialysis patients. Diabetes treatment should be preferentially selected for hemodialysis patients, and the introduction of HCV antiviral therapy should be determined based on the patient's clinical state.

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RESEARCH ARTICLE

Hepatitis E Virus in Cambodia: Prevalence among the General Population and Complete Genome Sequence of Genotype 4

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Data Availability Statement: Full-length sequence of the HEV genome obtained in this study files will be available from the DDBJ/EMBL/GenBank database (accession number LC042232). The dataset used in this manuscript is available upon request for an ethical restriction. To request the data, contact Professor Junko Tanaka, Department of Epidemiology, Infectious Disease Control and Prevention, Institute of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan.

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Abstract

Hepatitis E virus (HEV) is a growing public health problem in many countries. In this study, we investigated HEV seroprevalence among the general population in the Siem Reap province, Cambodia, and performed HEV genetic analysis with the aim to develop an HEV prevention strategy. This seroepidemiological cross-sectional study conducted from 2010 to 2014 included 868 participants from four different locations in Siem Reap province, Cambodia. They answered questionnaires and provided blood samples for the analysis of hepatitis virus infections. Among the participants (360 men and 508 women; age range, 7–90 years), the prevalence of anti-HEV IgG was 18.4% (95% confidence interval: 15.9–21.0); HEV RNA was detected in two participants (0.23%) and was classified as genotype 3 and 4. Full-length genome of the genotype 4 isolate, CVS-Sie10, was sequenced; it contained 7,222 nucleotides and three ORFs and demonstrated high sequence identity with the swine China isolates swGX40 (95.57%), SS19 (94.37%), and swDQ (91.94%). Multivariate logistic regression analysis revealed that men, elderly people, and house workers were risk groups significantly associated with the positivity for anti-HEV IgG. This is the first report on the detection of HEV genotype 4 in humans in Cambodia and on the complete genome sequence of HEV genotype 4 from this country. Our study demonstrates that new HEV infection cases occur frequently among the general population in Cambodia, and effective preventive measures are required.

Introduction

World Health Organization (WHO) statistics indicates that approximately 20 million people are hepatitis E virus (HEV)-infected, over 3 million have acute hepatitis E, and 70,000 die of

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hepatitis E every year worldwide [1, 2]. HEV is transmitted mainly through the fecal-oral route because of fecal contamination of drinking water; therefore, low sanitation standards increase the risk of HEV infection [1] which is a common cause of hepatitis outbreaks in the developing world [2]. In Cambodia, one of the developing countries in Asia, HEV infection can be an important health problem.

According to genome sequence, HEV has been classified into four genotypes; recently, new HEV genotype 5 has been identified in a wild boar in Japan [3]. HEV genotypes differ in their epidemiology and severity of infection. Genotype 1 is usually detected during hepatitis E outbreaks in developing countries in Asia, Africa, and South America; genotype 2 has been identified in Mexico, Chad, and Nigeria, while genotype 3 is more common in the developed countries, and genotype 4 has been found mainly in Asia, including Japan, China, and Taiwan [1, 4]. HEV genotype is one of the important risk factors associated with the disease severity [5–7]. In Cambodia, HEV RNA of genotype 3 has been detected in river water [8] and swine [9]; HEV genotype 1 has also been identified in swine [9]; in patients, anti-HEV IgG and IgM have been found [10, 11]. However, the full-length genome sequence of HEV isolated in Cambodia has not yet been submitted in the DDBJ/EMBL/GenBank database. We have been conducting a seroepidemiological survey on hepatitis virus infections among the general population in Cambodia since 2010 and have reported the seroprevalence and genotype distribution of hepatitis B and C virus among adults in this country [12]. In the current study conducted in collaboration with the Ministry of Health in Cambodia, we investigated the prevalence of HEV infection among the general population in Siem Reap province and sequenced full-length genome of the HEV isolate recovered from an HEV RNA-positive individual.

Materials and Methods

Study design

We conducted a cross-sectional study among the general population in Siem Reap province, Cambodia. Based on anticipated anti-HEV IgG rate of 15%, relative precision of 15%, confidence coefficient of 95% and the population size of approximately 3,000 (information from the village/commune chiefs), sample size was calculated to be 755. Therefore, intended sample size was determined to be 800.

Participants

Seroepidemiological surveys were performed eight times: in February and August, 2010; February and July, 2011; February and August, 2012; June, 2013; and June, 2014 among the general population of Chrey village, Sasar Sdam commune, Krabei Riel commune, and Rohal village in Siem Reap, a province in northwestern Cambodia. The proportion of main activity of general population in Cambodia was 51.8% of employed, 24.7% of student from the data of general population census of Cambodia 2008[13]. Then, we selected Sasar Sdam commune including elementary school according to the characteristic of the population, and three locations which have different background; Chrey was a new urban village, Krabei Riel was an old commune, and Rohal was a sightseeing craft village. On the day of the survey, a duty officer of the Ministry of Health, Cambodia, explained the study protocol to the participants or parents of elementary school students before they were enrolled in the study. The participants who were the residents of Chrey village (333 of total 2034; 16.4%), Krabei Riel commune (189 of total 447; 42.3%), and Rohal village (49 of total 100; 49.0%) were enrolled in this study by the village/commune chiefs. In Sasar Sdam commune, the participants included 240 of total 282 (85.1%) third-year elementary school students (as of 2011) and 57 people living around the school.

Ethical permission

This study, which was based on questionnaires and blood sample analyses for hepatitis virus infections, was approved by the Ethics Committees for epidemiological research of Hiroshima University, Japan, and the Ministry of Health, Cambodia. Written informed consent was obtained from all the participants or parents of elementary school students. We informed the participants about the results of serological tests for hepatitis virus infections and provided the pamphlet with healthcare information approximately six months later.

Questionnaires

Questionnaires were used to determine participants' characteristics such as age, sex, occupation, current health status, current periodic treatment, history of disease or a major injury, history of injection or infusion, operation, blood transfusion, tattoo and holes for pierced earrings.

Serological tests

About 10 ml of blood drawn from each participant was carefully centrifuged, and the serum samples were transported to Hiroshima University in Japan, where they were tested for hepatitis virus markers to determine the prevalence of hepatitis virus infection. HEV infection was identified by the presence of anti-HEV IgG and IgM antibodies detected using the enzyme immunoassay(EIA) with IgG/IgM anti-HEV EIA (Institute of Immunology Co., Ltd, Tokyo, Japan), and anti-HEV IgA was detected using IMMUNIS IgA anti-HEV EIA (Institute of Immunology). Hepatitis B virus (HBV) was identified based on seropositivity for hepatitis B surface antigen (HBsAg) detected by the reversed passive hemagglutination assay with Mycell II HBsAg (Institute of Immunology), hepatitis B surface antibody (anti-HBs) detected by passive hemagglutination (PHA) using Mycell II anti-HBs (Institute of Immunology) or Chemiluminescence Immunoassay (CLIA) using Architect Osabu (Abbott, Tokyo, Japan), or hepatitis B core antibody (anti-HBc) detected by PHA using Mycell anti-rHBc (Institute of Immunology) or CLIA with Architect HBc II (Abbott, Tokyo, Japan); a sample was considered HBV infection if either HBsAg or anti-HBc were detected with or without anti-HBs. Hepatitis C virus (HCV) infection was confirmed by seropositivity for anti-HCV antibodies by the particle agglutination test using Ortho HCV Ab PA test II (Ortho-Clinical Diagnostics, Inc., Tokyo, Japan). Hepatitis A virus (HAV) infection was detected by CLIA with Architect HAVAB- G (Abbott). HIV infection was determined by the gelatin-particle agglutination test with Serodia HIV-1/2 (Fujirebio Inc., Tokyo, Japan) and the presence of HIV RNA detected by reverse transcription (RT)-PCR [14].

Detection of HEV RNA

Nucleic acids were extracted from serum samples using Smitest EX-R & D (Medical & Biological Laboratories Co., Ltd. Nagano, Japan). HEV RNA was determined in each anti-HEV IgG-positive sample and in pooled sera of every 10 anti-HEV IgG-negative samples by nested RT-PCR with HE5 primers targeting ORF1 of the HEV genome [15].

HEV full-length genome sequencing

HEV genomic RNA was reverse transcribed and cDNA was amplified by PCR using primers specific for 12 overlapping regions in the HEV genome (Table 1). Reverse transcription and first-round PCR were conducted using the PrimeScript II High Fidelity One Step RT-PCR Kit (Takara Bio, Inc., Shiga, Japan); second-round PCR was conducted using PrimeSTAR GXL

Table 1. Hepatitis E Virus-specific oligonucleotide primers used in this study.

Primers	Stage-polarity	Nucleotide sequence (5'-3')
Primer set A	1st sense	GCAGACCACGTATGTGGTCG
	2nd sense	CCACGTATGTGGTCGACGCC
	1st antisense	ATRGACACATCATGRTTRTA
	2nd antisense	CCGGCACTRGARTCNCCCTC
Primer set B	1st sense	GCGGARGCNATGGCYCGYCA
	2nd sense	GGCATGACYCGGYTSTAYGC
	1st antisense	TARTCACGSCCRGAYTTYTC
	2nd antisense	CARCTRTARAGGCGYGTTAT
Primer set C	1st sense	AAGTCNACATTTCAYGCCGT
	2nd sense	GTGCAYATATGGGAYAGGCT
	1st antisense	CCTCCRATRAGRGARTGCCG
	2nd antisense	TGRACRCAGCGRTTRAACCT
Primer set D	1st sense	AYTWTGGGYAATAARACYTT
	2nd sense	CCCAGYTRGAGGYCAAYGG
	1st antisense	TTRGAYGCATTRACCAGCCA
	2nd antisense	CCRTCAGGRTARGTRTGRAG
Primer set E	1st sense	CTAAYCCYTTYTGYGGKGA
	2nd sense	CTYTAYACYCGSACYTGGTC
	1st antisense	AGYGANGGGGCCTCRTCRAT
	2nd antisense	GGTGTRTARGCTGCRAACCC
Primer set F	1st sense	AGYTTYGATGCYTGGGARCG
	2nd sense	CCAGCYATAGCYTGGTTYGA
	1st antisense	ATRCCNACCTCRCGRAGRAG
	2nd antisense	GCATCRACMACCACRCAYTT
Primer set G	1st sense	GTYCATGARGCYCARGGYGC
	2nd sense	TTYACTGAGACYACRATTAT
	1st antisense	TTYTCAATAGCRCGRAACCA
	2nd antisense	GTYTTRCTCCAYGCRGATAT
Primer set H	1st sense	AAYGTYACYACCTGYGAGCT
	2nd sense	GAGCTYGTRGAGGCYATGGT
	1st antisense	TGCGAAACAACATCMACACA
	2nd antisense	GCCACATTMGTTARCTTTCGCA
Primer set I	1st sense	AAYACYGTYTGGAAYATGGC
	2nd sense	GGGGATGAYTCTGTTGTRCT
	1st antisense	CGGCGAAGCCCCAGCTGGGG
	2nd antisense	AGCGGCGGGGCGCTGGGAYTG
Primer set J	1st sense	GTGGTTTCTGGGGTGACCGG
	2nd sense	TTCTCAGCCCTTCGCCCTCC
	1st antisense	TTAGTRTARGARTTYACAGG
	2nd antisense	CCATGRATRCARAGCATRAC
Primer set K	1st sense	TCYATYTCYTTYTGGCCYCA
	2nd sense	CCRACGTCYGTNGAYATGAA
	1st antisense	ACAGTRTCAGARACATACAT
	2nd antisense	AGCCARAGYACRTCATTRGC
Primer set L	1st sense	GTCTCAGCCAATGGCGAGC
	2nd sense	GTYGAGAAYGCYCAGCAGGA
	1st antisense	CARAATAAATCAATACTCCCG
	2nd antisense	TACCCACCTTCATYTTRAGACG

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DNA Polymerase (Takara Bio, Inc.). The 3′-Full RACE Core Set (Takara Bio, Inc.) was used to amplify core 3′ sequences. Final products were sequenced using a 3730xl DNA sequencer and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Molecular evolutionary analyses

The number of nucleotide substitutions per site was estimated by a six-parameter modeling method [16], and phylogenetic trees were constructed by the neighbor-joining method [17] based on the number of substitutions. To confirm the credibility of phylogenetic analyses, bootstrap resampling tests were carried out 1,000 times [18]. The analyses were performed using the GENETYX-MAC version 17 software (Genetyx Corporation, Tokyo, Japan).

Statistical analysis

The data were analyzed using the JMP 10 software (SAS Institute Inc., Cary, NC, USA). Proportions were estimated with the 95% confidence interval (CI); χ^2 test or Fisher's exact test and Mantel-extension test for trend were performed to evaluate the difference in the prevalence of viral markers among sex, age, residential, and occupational groups. Univariate analysis using χ^2 test or Fisher's exact test and multivariate logistic regression analysis were performed to identify potential risk factors for HEV infection by calculating odds ratios (ORs) and 95% CI. The explanatory variables were sex (reference: woman), age group (reference: 7–19 years old), location (reference: Chrey village), occupation (reference: farmer), and HBV or HCV infection (reference: positive). For all analyses, a p-value < 0.05 was considered statistically significant.

Results

Characteristics of the participants

Participants' characteristics are shown in Table 2. In total, there were 868 people, 360 men (41.5%) and 508 women (58.5%); age distribution was from 7 to 90 years (mean, 30.5 ± 18.8; median, 29 years) as of 2014. Among the participants, 38.4% lived in Chrey village, 34.2% in Sasar Sdam commune, 21.8% in Krabei Riel commune, and 5.6% in Rohal village, Siem Reap Province. In terms of occupation, most of the participants were students (40.1%), followed by farmers (33.2%), house workers (7.5%), office workers (6.9%), and craftsmen (2.4%). Other results of the questionnaire are shown in S1 Table.

HEV infection prevalence

The results of serologic testing are shown in Table 3. Overall, the prevalence of anti-HEV IgG was 18.4% (160/868; [95% CI: 15.9–21.0%]). Anti-HEV IgG positivity was significantly higher in men than in women (21.9% vs. 15.9%; $p = 0.0247$) and showed statistically significant correlation with older age ($p < 0.0001$). Anti-HEV IgG prevalence differed significantly among the four analyzed locations ($p < 0.0001$). There was also significant difference among occupational/professional groups ($p < 0.0001$) (Table 3).

HEV RNA was detected in two participants (0.23% [0–0.55%]) (Table 3). There were no significant differences in the positivity rate of HEV RNA among sex, age, residential, and occupational groups. Full-length HEV genome could be sequenced for one of the two samples and was classified as genotype 4. Another HEV RNA positive isolate was classified as genotype 3 based on partial ORF1 sequence with HE5 primers [15].

The two HEV RNA-positive participants were analyzed for other hepatitis viruses and HIV (Table 4). They were found positive for anti-HEV IgG and IgM, and one of them was positive

Table 2. Characteristics of participants.

	Characteristics	Total		Men		Women	
		N	(%)	N	(%)	N	(%)
Age group(yr)	7–19	330	(38.0)	146	(40.6)	184	(36.2)
	20–29	118	(13.6)	56	(15.6)	62	(12.2)
	30–39	136	(15.7)	48	(13.3)	88	(17.3)
	40–49	124	(14.3)	61	(16.9)	63	(12.4)
	50–59	85	(9.8)	31	(8.6)	54	(10.6)
	60–90	75	(8.6)	18	(5.0)	57	(11.2)
Location	Chrey village	333	(38.4)	122	(33.9)	211	(41.5)
	Sasar Sdam commune	297	(34.2)	126	(35.0)	171	(33.7)
	Krabei Riel commune	189	(21.8)	70	(19.4)	119	(23.4)
	Rohal village	49	(5.6)	42	(11.7)	7	(1.4)
Occupation	student	348	(40.1)	152	(42.2)	196	(38.6)
	farmer	288	(33.2)	118	(32.8)	170	(33.5)
	house worker	65	(7.5)	1	(0.3)	64	(12.6)
	office worker	60	(6.9)	25	(6.9)	35	(6.9)
	craftsman	21	(2.4)	10	(2.8)	11	(2.2)
	others	86	(9.9)	54	(15.0)	32	(6.3)
Total		868	(100.0)	360	(41.5)	508	(58.5)

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for anti-HEV IgA, as well as anti-HBs and anti-HBc, indicating previous HBV infection. Moreover, one of them was positive for anti-HIV antibody, but HIV RNA was not detected.

Full-length sequence of the HEV genome

The full-length genome sequence of HEV isolate was recovered from a 39-year-old man at the time of the survey. The isolate designated as CVS-Sie10 (DDBJ/EMBL/GenBank accession number LC042232) had the genome of 7,222 nucleotides (nt) containing three ORFs: ORF1 [1–5,115 nt; 1,705 amino acids (aa)], ORF2 (5,115–7,136 nt; 674 aa), and ORF3 (5,143–5,484 nt; 114 aa), a 3′ UTR, and a poly-A tail.

The alignment of the CVS-Sie10 genome with published HEV genotype 4 sequences showed that this isolate was close to the swine China isolates swGX40, SS19, and swDQ with sequence identities of 95.57%, 94.37%, and 91.94%, respectively (Table 5), and the same length of ORF1, ORF2, and ORF3. CVS-Sie10 displayed weak homology with other HEV genotypes. Thus, it demonstrated 76.99%, 76.83%, 78.09%, and 79.34% identity with the Burma isolate of genotype 1, the Mexico isolate of genotype 2, the HEV-US2 isolate of genotype 3, and the JBOAR135--Shiz09 isolate of genotype 5, respectively.

Based on full-length genome sequences of the CVS-Sie10 and other HEV isolates, we constructed a phylogenetic tree (Fig 1), which showed that CVS-Sie10 clustered on a branch separate from the other genotype 4 sequences, and close to the China isolates swGX40, SS19, and swDQ.

Potential risk factors for HEV infection

The odds ratios and *p*-values of potential risk factors for anti-HEV IgG positivity in univariate analysis and multivariate logistic regression model are shown in Table 6. In multivariate analysis, men (AOR: 1.9 [1.2–2.8] $p = 0.0025$) and older age (if the 7–19-year-old population group is taken as baseline) were significantly associated with anti-HEV IgG seropositivity. There were no significant differences among the four locations. The odds ratio of anti-HEV IgG seropositivity

Table 3. Sex-, age-, location- and occupation-specific prevalence of anti-HEV IgG and HEV RNA among the general population in Cambodia.

			anti-HEV IgG positive				HEV RNA positive			
			N	(%)	[95% CI]	*p*-Value	N	(%)	[95% CI]	*p*-Value
Total		868	160	(18.4)	[15.9–21.0]		2	(0.23)	[0–0.55]	
Sex	Man	360	79	(21.9)	[17.7–26.2]	0.0247*[a]	1	(0.28)	[0–0.82]	0.8065[a]
	Woman	508	81	(15.9)	[12.8–19.1]		1	(0.20)	[0–0.58]	
Age group	7–19	330	19	(5.8)	[3.2–8.3]	<0.0001*[c]	0	(0.0)	[0–1.1]	0.3839[b]
	20–29	118	25	(21.2)	[13.8–28.6]		0	(0.0)	[0–3.1]	
	30–39	136	32	(23.5)	[16.4–30.7]		1	(0.74)	[0–2.2]	
	40–49	124	36	(29.0)	[21.0–37.0]		1	(0.81)	[0–2.4]	
	50–59	85	30	(35.3)	[25.1–45.5]		0	(0.0)	[0–4.3]	
	60–90	75	18	(24.0)	[14.3–33.7]		0	(0.0)	[0–4.9]	
Location	CV	333	74	(22.2)	[17.8–26.7]	<0.0001*[a]	1	(0.30)	[0–0.89]	1.0000[b]
	SC	297	29	(9.8)	[6.4–13.1]		1	(0.34)	[0–1.0]	
	KC	189	47	(24.9)	[18.7–31.0]		0	(0.0)	[0–2.0]	
	RV	49	10	(20.4)	[9.1–31.7]		0	(0.0)	[0–7.5]	
Occupation	farmer	288	62	(21.5)	[16.8–26.3]	<0.0001*[a]	0	(0.0)	[0–1.3]	0.0712[b]
	student	348	24	(6.9)	[4.2–9.6]		0	(0.0)	[0–1.1]	
	house worker	65	21	(32.3)	[20.9–43.7]		1	(1.5)	[0–4.5]	
	office worker	60	18	(30.0)	[18.4–41.6]		0	(0.0)	[0–6.1]	
	craftsman	21	7	(33.3)	[13.2–53.5]		0	(0.0)	[0–17.6]	
	others	86	28	(32.6)	[22.7–42.5]		1	(1.2)	[0–3.4]	

CV: Chrey village, SC: Sasar Sdam commune, KC: Krabei Riel commune, RV: Rohal village, CI: Confidence Interval

* statistically significant variables.

[a] $\chi 2$ test

[b] Fisher's exact test

[c] Mantel-extension test for trend

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for house workers was more than twice higher than that for farmers (AOR: 2.3 [1.2–4.5]; $p = 0.0109$), and that for others was significantly higher than farmers (AOR: 1.8 [1.0–3.2]; $p = 0.0464$). On the other hand, HBV and HCV infections were not associated with anti-HEV IgG seropositivity.

Discussion

In this study, we performed a seroepidemiological survey for HEV infection in Cambodia. As a result, we identified the first human case of HEV genotype 4 in Cambodia and performed

Table 4. HEV RNA positives among the general population in Cambodia.

No	Sex	Age[a]	year	anti-HEV IgG		anti-HEV IgM		anti-HEV IgA		HEV RNA	HEV genotype	HBsAg	anti-HBs	anti-HBc	HBV DNA	anti-HCV	HCV RNA	anti-HAV	anti-HIV	HIV RNA
					COI		COI		COI											
1	M	39	2010	+	23.4	+	1.2	-	0.7	+	4	-	+	+	-	-	-	+	+	-
2	F	33	2011	+	6.8	+	2.3	+	3.3	+	3	-	+	+	-	-	-	+	-	NT

M: Male, F: Female, NT: Not tested, COI: Cut off index

[a] at survey

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Table 5. Identity of full-genome sequences of HEV isolates known.

Genotype	Isolate name	Accession number	Source		Nucleotides length	Identity with CVS-Sie10 (%)	Amino acids length		
			Country	host			ORF1	ORF2	ORF3
4	CVS-Sie10	LC042232	Cambodia (Siem Reap)	human	7222		1705	674	114
	swGX40	EU676172	China (Guangxi)	swine	7269	95.57	1705	674	114
	SS19	JX855794	China (Guangdong)	swine	7233	94.37	1705	674	114
	swDQ	DQ279091	China	swine	7234	91.94	1705	674	114
	JYK-Tok03C	AB291964	Japan (Tokyo)	human	7244	87.44	1705	674	114
	HEVN2	AB253420	Japan (Okinawa)	human	7253	87.40	1707	674	114
	EChZ20	HM439284	China (eastern China)	human	7229	86.59	1704	674	114
	W2-5	JQ655736	China (Beijing)	human	7261	85.50	1706	674	114
	JYI-ChiSai01C	AB197674	China (Shanghai)	human	7260	85.00	1706	674	114
1	Burma	M73218	Burma	human	7207	76.99	1693	660	123
2	Mexico	M74506	Mexico	human	7180	76.83	1691	659	123
3	HEV-US2	AF060669	the United States	human	7277	78.09	1708	660	122
5	JBOAR135-Shiz09	AB573435	Japan (Shizuoka)	wild boar	7267	79.34	1708	674	112

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full-length genome sequencing of the isolate. One case of HEV genotype 3 was also detected. In developing countries, HEV infection is usually caused by genotype 1 viruses [1]; however, the data obtained in our study shows different genotype. In previous studies in Cambodia, HEV genotype 3 was detected in river water [8], and genotypes 1 and 3 were recovered from swine [9]. Partial sequences of HEV genotype 1 (accession number: DQ145797) and genotype 3 (accession number: DQ145792) from humans have been determined, but genotype 4 has not been reported. In this study, we sequenced, for the first time, the full-length genome of human HEV genotype 4 isolated in Cambodia.

Two HEV RNA-carrying participants were also positive for anti-HEV IgM and had a prior history of HBV infection as evidenced by the presence of anti-HBs and anti-HBc antibodies; one of the participants was also anti-HIV-positive. The detection of anti-HEV IgM indicates that both individuals had early stages of HEV infection. HEV genotype 4 has been reported to cause higher rate of aggravation from viral infection than genotype 3 [5, 6, 19], however, according to their answers to our questionnaire, both participants (genotype 3 and genotype 4) did not receive periodic treatment in hospitals and did not have serious problems with health. Moreover, they were confirmed asymptomatic during the interview 6 months after a blood test. Both of them had a prior history of HBV infection, which is not a rare case for Cambodia, where adult population has high anti-HBc positive rate of 38.5% [12]. Our multivariate analysis revealed no association between HEV and HBV infections.

Aggravation due to HEV infection is infrequent, and was not observed in this study. HEV genotype 4 is more likely to cause aggravation, and sometimes cause death [20, 21]; in recent years, the risk of HEV triggering chronic hepatitis in immunocompromised patients, including HIV carriers [22] and organ transplant recipients [23, 24] has been reported. These data underscore the importance of investigating the prevalence and performing genetic analysis of HEV infection in Cambodia, which is the part of Asia believed to be heavily affected by HEV infection, and the necessity of developing preventive measures against HEV spread.

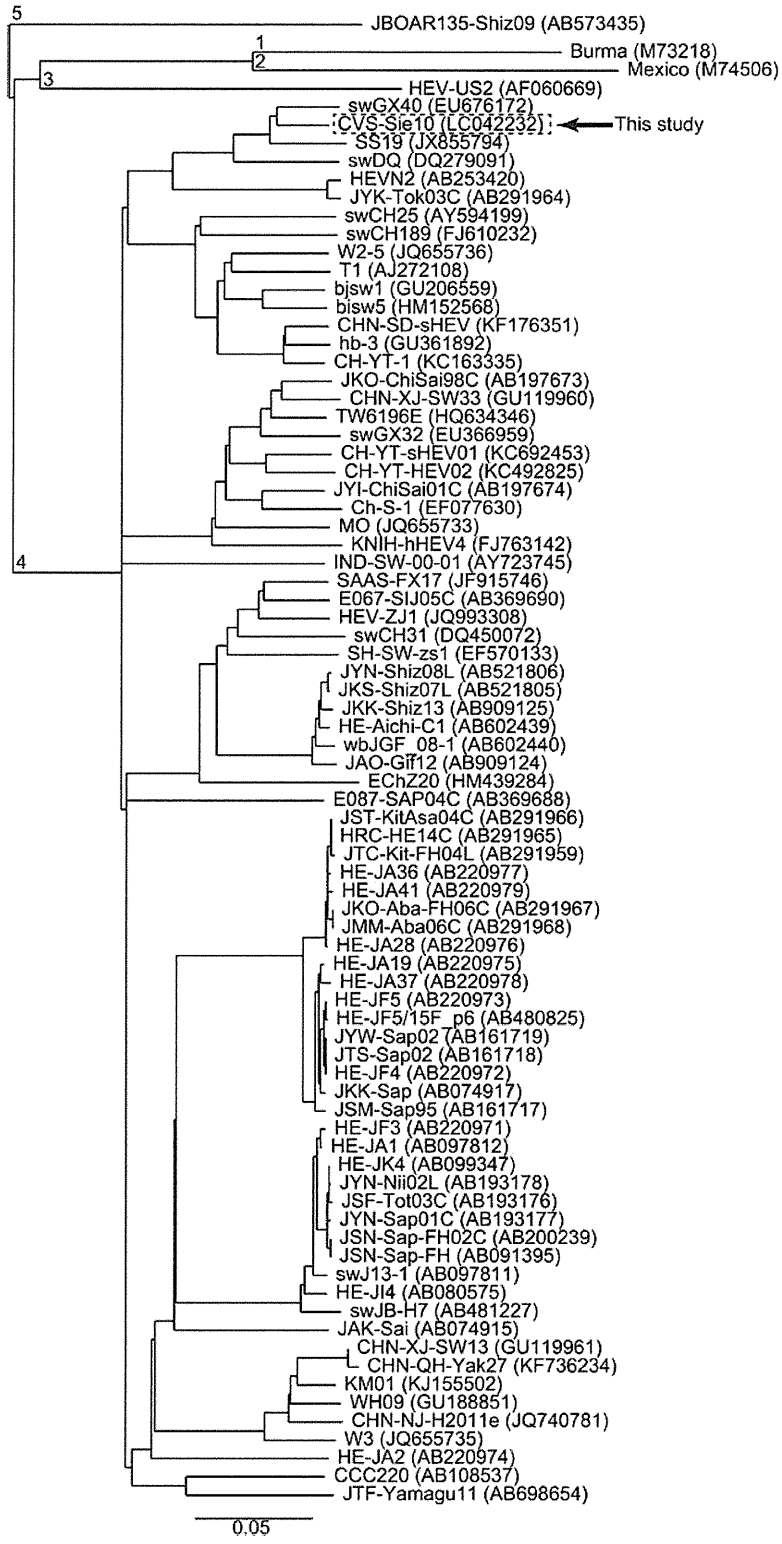
5
JBOAR135-Shiz09 (AB573435)
1
Burma (M73218)
2
Mexico (M74506)
3
HEV-US2 (AF060669)
swGX40 (EU676172)
CVS-Sie10 (LC042232)
This study
SS19 (JX855794)
swDQ (DQ279091)
HEVN2 (AB253420)
JYK-Tok03C (AB291964)
swCH25 (AY594199)
swCH189 (FJ610232)
W2-5 (JQ655736)
T1 (AJ272108)
bjsw1 (GU206559)
bisw5 (HM152568)
CHN-SD-sHEV (KF176351)
hb-3 (GU361892)
CH-YT-1 (KC163335)
JKO-ChiSai98C (AB197673)
CHN-XJ-SW33 (GU119960)
TW6196E (HQ634346)
swGX32 (EU366959)
CH-YT-sHEV01 (KC692453)
CH-YT-HEV02 (KC492825)
JYI-ChiSai01C (AB197674)
Ch-S-1 (EF077630)
MO (JQ655733)
KNIH-hHEV4 (FJ763142)
4
IND-SW-00-01 (AY723745)
SAAS-FX17 (JF915746)
E067-SIJ05C (AB369690)
HEV-ZJ1 (JQ993308)
swCH31 (DQ450072)
SH-SW-zs1 (EF570133)
JYN-Shiz08L (AB521806)
JKS-Shiz07L (AB521805)
JKK-Shiz13 (AB909125)
HE-Aichi-C1 (AB602439)
wbJGF_08-1 (AB602440)
JAO-Gif12 (AB909124)
EChZ20 (HM439284)
E087-SAP04C (AB369688)
JST-KitAsa04C (AB291966)
HRC-HE14C (AB291965)
JTC-Kit-FH04L (AB291959)
HE-JA36 (AB220977)
HE-JA41 (AB220979)
JKO-Aba-FH06C (AB291967)
JMM-Aba06C (AB291968)
HE-JA28 (AB220976)
HE-JA19 (AB220975)
HE-JA37 (AB220978)
HE-JF5 (AB220973)
HE-JF5/15F_p6 (AB480825)
JYW-Sap02 (AB161719)
JTS-Sap02 (AB161718)
HE-JF4 (AB220972)
JKK-Sap (AB074917)
JSM-Sap95 (AB161717)
HE-JF3 (AB220971)
HE-JA1 (AB097812)
HE-JK4 (AB099347)
JYN-Nii02L (AB193178)
JSF-Tot03C (AB193176)
JYN-Sap01C (AB193177)
JSN-Sap-FH02C (AB200239)
JSN-Sap-FH (AB091395)
swJ13-1 (AB097811)
HE-JI4 (AB080575)
swJB-H7 (AB481227)
JAK-Sai (AB074915)
CHN-XJ-SW13 (GU119961)
CHN-QH-Yak27 (KF736234)
KM01 (KJ155502)
WH09 (GU188851)
CHN-NJ-H2011e (JQ740781)
W3 (JQ655735)
HE-JA2 (AB220974)
CCC220 (AB108537)
JTF-Yamagu11 (AB698654)
0.05

Fig 1. Phylogenetic tree constructed based on HEV full-length genomes using the neighbor-joining method. Each of HEV genotypes 1, 2, 3, and 5 is represented by a single isolate, while for genotype 4, all the isolates with reported complete or near-complete genome sequence are presented. GenBank accession numbers are shown in parentheses; scale bar indicates nucleotide substitutions per site.

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In our survey, the overall rate of anti-HEV IgG positivity among 868 participants was 18.4%. There has been no prior study on the prevalence of HEV infection in the general population in Cambodia; however, the rate of anti-HEV IgG positivity in patients with high aspartate transaminase and alanine transaminase levels was determined as 5.5% [10], and the rate of anti-HEV IgM positivity in feverish patients was 11.1% [11]. Other studies have indicated that the rate of anti-HEV IgG positivity among blood donors in Japan was 3.4% [25] and in the general population of the Mekong River basin in Vietnam it was 9% [26]; among the general population of the East China Sea coast in the same country it was 28.1% (143/509 [95% CI: 24.2–32.0%]) (our unpublished data). In China, Taiwan, India, and Thailand, HEV IgG

Table 6. Univariate and multivariate analysis of positivity for anti-HEV IgG among the general population in Cambodia.

			anti-HEV IgG					
			Univariate analysis[a]			Multivariate analysis[b]		
		N	OR	[95% CI]	*p*-Value	AOR	[95% CI]	*p*-Value
Sex	Man	360	1.5	[1.1–2.1]	0.0247*	1.9	[1.2–2.8]	0.0025*
	Woman	508	1			1		
Age group(yr)	7–19	330	1			1		
	20–29	118	4.4	[2.3–8.3]	<0.0001*	5.7	[1.7–17.8]	0.0037*
	30–39	136	5.0	[2.7–9.3]	<0.0001*	7.1	[1.8–26.2]	0.0038*
	40–49	124	6.7	[3.7–12.3]	<0.0001*	9.2	[2.4–34.1]	0.0010*
	50–59	85	8.9	[4.7–17.0]	<0.0001*	12.3	[3.1–46.3]	0.0002*
	60–90	75	5.2	[2.6–10.4]	<0.0001*	6.7	[1.6–26.3]	0.0068*
Location	CV	333	1			1		
	SC	297	0.38	[0.24–0.60]	<0.0001*	0.95	[0.51–1.8]	0.8708
	KC	189	1.2	[0.76–1.8]	0.4912	1.2	[0.71–1.9]	0.5667
	RV	49	0.90	[0.43–1.9]	0.7747	0.56	[0.23–1.2]	0.1716
Occupation	farmer	288	1			1		
	student	348	0.27	[0.16–0.45]	<0.0001*	1.9	[0.58–5.8]	0.2643
	house worker	65	1.7	[0.96–3.1]	0.0642	2.3	[1.2–4.5]	0.0109*
	office worker	60	1.6	[0.84–2.9]	0.1559	1.5	[0.75–3.1]	0.2231
	craftsman	21	1.8	[0.71–4.7]	0.2098	2.5	[0.87–6.8]	0.0742
	others	86	1.8	[1.0–3.0]	0.0357*	1.8	[1.0–3.2]	0.0464*
HBV infection	positive	247	2.0	[1.4–2.9]	<0.0001*	1.1	[0.75–1.7]	0.5593
	negative	621	1			1		
HCV infection	positive	34	1.9	[0.89–4.1]	0.0921	1.2	[0.52–2.7]	0.6224
	negative	834	1			1		

OR: Odds Ratio, AOR: Adjusted Odds Ratio, CI: Confidence Interval, CV:Chrey village, SC: Sasar Sdam commune, KC: Krabei Riel commune, RV: Rohal village

[a] χ^2 test or Fisher's exact test

[b] Logistic regression analysis: $R^2 = 0.1113$, Model p<0.0001*, N = 868

* statistically significant variables

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