Case Report: Hepatitis E in Singapore: A Case-Series and Viral Phylogenetics Study

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Abstract. The incidence of hepatitis E in Singapore appears to be increasing. A retrospective case-series study of patients diagnosed with hepatitis E in a tertiary hospital from 2009 to 2013 was conducted. Of 16 cases, eight (50%) were solid-organ transplant recipients (SOTRs), and 14 (88%) were found infected by genotype 3 hepatitis E virus (HEV-3). Bayesian inferences based on HEV subgenomic sequences from seven cases suggest that HEV-3 strains were introduced to Singapore as two principal lineages. Within limitations of the study, it can be inferred that one lineage, in the 3efg clade, emerged about 83 years ago, probably originating from Japan, whereas the other, in the 3abchij clade, emerged about 40 years ago, from the United States. Establishment and subsequent transmissions of strains from these two lineages likely contribute to the current endemicity of hepatitis E in Singapore.

INTRODUCTION

Hepatitis E is caused by hepatitis E virus (HEV), a single-stranded RNA virus belonging to the *Hepeviridae* family. Epidemic and sporadic disease is common in south and central Asia, and Africa where many localities lack adequate sanitation and easy access to potable water. Clinical attack rates are highest in young adults (i.e., in the 15- to 49-year age group). Although disease is frequently self-resolving, pregnant women are particularly disposed to acute liver failure, with ensuing high rates of maternal mortality, miscarriages, and neonatal deaths. These features typify classical hepatitis E. It is associated with infection by HEV belonging primarily to genotype 1 (HEV-1), and more rarely, genotype 2.¹

Sporadic disease is also encountered where waterborne hepatitis E outbreaks do not occur, such as Europe, North America, and east Asia. Clinical disease in these relatively affluent regions, contrasting with that in developing countries, is noted most frequently among older adults (i.e., aged > 45 years), with acute liver failure observed mainly in the elderly (> 60 years). This form of hepatitis E is associated with infection by HEV genotype 3 (HEV-3) or 4 (HEV-4). Infection in solid-organ transplant recipients (SOTRs) can turn chronic, leading to cirrhosis. Hepatitis E among SOTRs is predominantly associated with HEV-3,² with very few cases ascribable to HEV-4.³

Singapore is situated in southeast Asia, wherein are locales that have experienced waterborne hepatitis E outbreaks, such as Burma,⁴ Vietnam,⁵ and Kalimantan, and Java in Indonesia.^{6,7} Nonetheless, there is a zone⁸ stretching from Singapore to Peninsular Malaysia,⁹ Thailand,¹⁰ Cambodia,¹¹ Laos,¹² and to the Philippines,¹³ where hepatitis E is not epidemic even though sporadic disease has been encountered. Up to the end of the previous century, hepatitis E in Singapore was predominantly a disease imported from south Asia.¹⁴ Between 2000 and 2011, however, a > 20-fold rise in notifications of laboratory-confirmed acute hepatitis E from persons with no history of recent travel abroad was

noted.¹⁵ These findings, which point to emergence of indigenously acquired hepatitis E, are similar to what have been observed over the past decade in east Asia, viz., Japan,¹⁶ Taiwan,¹⁷ South Korea,¹⁸ China,¹⁹ and Hong Kong Special Administrative Region.²⁰ In most of these east Asian countries, HEV-4 is the prevailing genotype, whereas in Japan, both genotypes 3 and 4 circulate.¹⁶

Recently, we encountered a fatal case of chronic hepatitis E in a SOTR.²¹ The genotype of HEV characterized from that patient was neither HEV-1 nor HEV-4, but HEV-3. These observations prompted a review of records of patients diagnosed with hepatitis E in the Department of Gastroenterology and Hepatology, Singapore General Hospital, a major tertiary health-care center in Singapore. We extended the study to inquire into the molecular epidemiology and evolutionary history of HEV strains infecting these patients.

MATERIALS AND METHODS

Study patients. Patients referred from January 2009 to December 2013 identified with hepatitis E were included in this retrospective study. A case of hepatitis E was defined as a patient with jaundice or abnormal liver biochemistries whose serum was positive for HEV RNA, regardless of positivity for anti-HEV IgM or IgG.

Anti-HEV IgM and IgG detection. Sera were tested in the Department of Pathology by immunoassay using the anti-HEV IgM enzyme-linked immunosorbent assay (ELISA) 3.0 and the anti-HEV IgG ELISA (MP Biomedicals, Singapore).¹⁵

HEV genotypic analyses. Study sera were processed for nucleic acids extraction, reverse transcription, and nested polymerase chain reaction (PCR).²² Genotyping was conducted by comparing study sample sequences amplified from a 164-nt segment that overlaps open reading frames (ORFs) 2 and 3 of the HEV genome with corresponding sequences representing all four HEV genotypes downloaded from GenBank. Phylogenetic analyses were done by sequencing a 691-nt segment amplified from ORF 2.²³ The Singapore sequences were compared against 113 global HEV-3 sequences (see Supplemental Table 1) covering nt positions 6,469 to 7,115, aligned using ClustalX (version 2.1),²⁴ and analyzed using BEAST (version 1.8.2).²⁵

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MEGA6 (build 6140212) was used with the Kimura 2-parameter model to calculate the genetic distance between all sequences used in this analysis to determine how related the Singapore sequences were to the global sequences.²⁶

All parameters in the discrete trait model were examined individually using a stepping stone analysis²⁷ to determine the best parameters to use. Based on these analyses, discrete trait models were run using the Kimura 2-parameter substitution model with four gamma rate categories and invariant sites. Fixed clock rate of 1.13×10^{-3} substitution per site per year²⁸ was assumed, based on our earlier modeling of the evolution of HEV ORF 2.²⁶ We used geographic locations from where the sequences originated as discrete variables to estimate the possible countries of origin for the Singapore sequences, with discrete trait model (<http://beast-classic.googlecode.com/files/ARv2.0.1.pdf>) and the continuous time Markov chain rate reference prior distribution and the coalescent Gaussian Markov Random Field Bayesian Skyride tree prior.²⁹ The discrete location trait uses the symmetric substitution model but with Bayesian stochastic search variable selection to infer the social network and the strict clock with the default rate. At least 2×10^8 iterations were made to ensure an effective sample size of > 500 . Model selection was also done using the stepping stone algorithm with default values,²⁷ with 10% burn-in applied to each run. The phylogenetic tree was created using TreeAnnotator (version 1.8.0; Ashworth Laboratories, Edinburgh, United Kingdom) and visualized with FigTree (version 1.4.0; Ashworth Laboratories, Edinburgh, United Kingdom). Two trees were created: one contains information about the most probable location for each branch within the tree and the associated location probability; the other contains the most probable location for each branch within the tree and the posterior probability.

RESULTS

Demographic and clinical features. A total of 16 cases were HEV-RNA positive. Of these, eight (50%) were SOTRs,

and the rest were not. Follow-up ranged from 74 to 1,480 days (mean = 572 days). Characteristics of the cases are summarized in Tables 1 and 2.

SOTR cases. Of the eight SOTRs, four had received allogeneic liver transplants (Patients 1–4) and the other four (Patients 5–7, 9) had received allogeneic kidney transplants (Table 1). The patient who was the subject of our earlier case report²¹ is Patient 3. Among the SOTRs, the median age was 62.5 years, and six (75%) were women. All the SOTR cases were anicteric, with abnormal liver biochemistries noted during posttransplantation follow-up visits. The interval between transplantation and hepatitis E ranged from 3 to 103 m (mean = 48 m). The median peak alanine aminotransferase (ALT) activity was 183.5 IU/L. Liver biopsy was conducted for five SOTRs: periportal and lobular inflammation were consistently observed, and fibrosis was noted in two cases (Patients 3 and 4). Anti-HEV IgM and IgG were variably detected among seven, with one case (Patient 7) being repeatedly seronegative. One patient died of sepsis and another from myocardial infarct.

Non-SOTR cases. Among the eight non-SOTRs, three cases were receiving chemotherapy for lymphoma (Patients 8 and 11) or leukemia (Patient 16) (Table 2). Patients 8 and 11 remained anicteric. Anti-HEV IgM and IgG were variably detected. Patient 16 was jaundiced, and liver biopsy showed features of fibrosis; he had a positive travel history to Vietnam, and subsequently died of sepsis. Two other cases (Patients 10 and 14) had been on steroid therapy when they presented with jaundice (Table 2). Anti-HEV IgM was detected in acute-phase sera, followed by anti-HEV IgG in convalescent sera. The liver biopsy of Patient 10 showed fibrosis. The rest of the three non-SOTRs (Patients 12, 13, and 15) were apparently healthy until the onset of jaundice (Table 2). Illness spontaneously resolved in all. Anti-HEV IgG but not IgM was detected in acute-phase sera; anti-HEV IgM remained undetected in convalescent sera. For non-SOTRs as a whole, the median age was 53.5 years, two were females, all were jaundiced, and the median peak ALT activity was 1,253.5 IU/L.

TABLE 1
Characteristics of HEV-infected SOTRs*

Characteristics of HEV-infected donors									
Patient	Sex/age	Underlying disease requiring transplant	Immunosuppressive given at time of diagnosis	Time between transplant and diagnosis (m)	Maximum ALT (IU/L)	Liver histology		HEV genotype	Country traveled†
						Fibrosis	Other findings		
Liver transplant recipients									
1	M/62	Primary biliary cirrhosis	Tacrolimus	50	123	No	Portal inflammation	3	None
2	F/64	Autoimmune hepatitis	MMF, tacrolimus	16	252	NA‡	NA‡	3	None
3§	M/49	Chronic hepatitis B; hepatocellular carcinoma	Tacrolimus, prednisolone	3	129	Yes	Lobular inflammation; centrilobular cholestasis with necrosis	3	China
4§	F/63	Primary biliary cirrhosis	Tacrolimus, prednisolone, azathioprine	80	189	Yes	Portal inflammation	3	None
Kidney transplant recipients									
5	F/65	ESRF	Rituximab, everolimus	84	178	NA‡	NA‡	3	None
6	F/44	Glomerulonephritis	Tacrolimus, Prensisolone	103	228	No	Portal inflammation	3	None
7	F/66	ESRF	Tacrolimus, prednisolone ciclosporin	14	352	No	Portal and lobular inflammation	Unknown	None
9	F/55	ESRF	MMF, tacrolimus, prednisolone	39	136	NA‡	NA‡	3	Japan

ALT = alanine aminotransferase; ESRF = end-stage renal failure; F = female; HEV = hepatitis E virus; M = male; MMF = mycophenolate mofetil; NA = not applicable; SOTRs = solid-organ transplant recipients.

*All Singapore citizens, and none jaundiced at time of hepatitis E diagnosis.

†Within 2 months before hepatitis E diagnosis.

‡Liver biopsy not done.

§Died (of causes not related to HEV infection).

TABLE 2
Characteristics of HEV-infected non-SOTRs*

Characteristics of HEV-infected non-COVID-19 patients									
Patient	Sex/age	Underlying disease requiring immunosuppression	Immunosuppressive therapy at time of diagnosis	Jaundice	Maximum ALT (IU/L)	Liver histology		HEV genotype	Country traveled†
						Fibrosis	Other findings		
Immunosuppressed									
8‡	M/25	Natural killer T-cell lymphoma	Dexamethasone, methotrexate, ifosfamide, asparaginase, etoposide	No	1,570	NA§	NA§	3	None
10	F/53	Autoimmune hepatitis	Prednisolone	Yes	1,956	Yes	Portal and lobular inflammation	3	None
11	M/55	Diffuse large B-cell lymphoma	Rituximab, cyclophosphamide, daunorubicin, vincristine, prednisone	No	937	NA§	NA§	Unknown	China
14	F/67	Myasthenia gravis	Azathioprine, prednisolone	Yes	66	NA§	NA§	3	None
16‡	M/54	Chronic lymphocytic leukemia	Fludarabine, cyclosporine, melphalan, budesonide	Yes	348	Yes	Portal and lobular inflammation; ductopenia	3	Vietnam
Immunocompetent									
12¶	M/46	None	NA	Yes	5,378	NA§	NA§	3	India
13¶	M/35	None	NA	Yes	149	NA§	NA§	3	India
15¶	M/69	None	NA	Yes	2,636	NA§	NA§	3	Thailand

ALT = alanine aminotransferase; F = female; HEV = hepatitis E virus; M = male; NA = not applicable; SOTRs = solid-organ transplant recipients.

*All Singapore citizens except Patient 16, a Vietnamese.

†Within 2 months before hepatitis E diagnosis.

‡Died (of causes not related to HEV infection).

§Liver biopsy not done.

¶Spontaneous resolution of HEV infection.

Differences in demographic and clinical features.

Among SOTRs as compared with non-SOTRs, the difference in the median age was not significant and the median peak ALT activity was lower ($P < 0.05$) (significance of difference calculated using the Wilcoxon–Mann–Whitney test); and the proportion with females was higher, whereas the proportion with jaundice was lower (both $P < 0.01$, using the Fisher's exact test).

HEV phylogenetics and temporal dynamics. *Genotype distribution.* HEV cDNA could be amplified from the 16 cases. Sequences from the ORF 2/3 region were characterized from 14 patients and determined to belong to HEV-3.

Phylogenetic relationships. Seven (50%) of the 14 sera permitted further PCR-sequencing of the ORF 2 region for phylogenetic construction. Figure 1 shows a tree with information on the most probable location for each branch within the tree and the associated location probability. Supplemental Figure 1 shows the most probable location for each branch of the tree and the posterior probability. The sequences grouped as three lineages within two major clades of human and porcine sequences. Sequences from Patients 5, 8, 9, and 15 constituted Lineage I, and is in the 3efg clade. Lineage II, formed by sequences from Patients 3 and 14, and Lineage III, comprising the sequence from Patient 16, are in the 3abchij clade (Figure 1). The mean distance among Lineage I sequences was 0.094 (range 0–0.126), and among Lineage II sequences was 0.021.

Divergence times. The best clock as determined by the stepping stone algorithm for the Bayesian runs was the strict clock, but outcomes from the lognormal and random local clocks were similar (data not shown). The time to most recent ancestor of all the 120 HEV-3 sequences examined was 164 years ago (95% highest posterior density [HPD], 146–182 years). The TMRCA for Lineage I was 83 years ago (95% HPD, 73–94 years), for Lineage II was 40 years ago (95% HPD, 32–48 years), and for Lineage III was 23 years ago (95% HPD, 14–30 years). Two intro-

ductions are inferred because the estimated temporal separation between Lineages I and II is at least 81 years (the time from the root to the Lineage I most recent common ancestor).

Origin of introductions. A discrete trait Bayesian model in which the country of sample collection is the trait indicates that Lineage I, the country with the highest location probability at the parent node for the Singapore sequences is Japan with a probability of 0.69, and Lineages II and III originated from the United States, with location probabilities of 0.84 and 0.81, respectively.

DISCUSSION

Nonimported, sporadic hepatitis E is increasingly being recognized in east and southeast Asia.^{9–20} It bears characteristics resembling hepatitis E that is indigenous to Europe and the United States, that is, higher clinical attack rates in older rather than young adults, and excess case-fatality rates among elderly men and women but not pregnant women.¹ Although in the west chronic HEV infection also has been found among SOTRs,² up to recently^{3,30} it is unknown if this phenomenon extends to SOTRs in Asia. Prompted by findings from our first SOTR patient,²¹ we have identified seven more SOTRs with hepatitis E (Table 1).

In addition to the SOTRs, we document hepatitis E among five patients who were not SOTRs but were otherwise immunosuppressed, and three non-SOTR patients who were previously healthy (Table 2). We had relied on PCR, not serology, to especially identify SOTRs with hepatitis E, in light of reports that anti-HEV IgM and IgG in SOTRs and other immunocompromised persons may not always be serologically detectable.^{31,32} Such reliance likely restricted the number of non-SOTR cases of hepatitis E that could be enrolled.

Compared with non-SOTRs, there appears to be a trend for the SOTR cases to be older and female, and the

FIGURE 1. Bayesian phylogenetic tree from discrete trait model. Sequence IDs contain information about country of origin, host, GenBank accession number, and year of collection. Branches are labeled with highest country location probability. Nodes are labeled with the two-letter code for country with the highest probability. Country abbreviations: BR = Brazil; CA = Canada; CN = China; DE = Germany; ES = Spain; FR = France; HU = Hungary; JP = Japan; KR = Kyrgyzstan; MN = Mongolia; SE = Sweden; SG = Singapore; TH = Thailand; UK = United Kingdom; US = United States. Host abbreviations: Dr = deer; Hu = human; Mg = mongoose; Sw = swine; Wb = wild boar; xx = unknown. Singapore sequences belonging to lineages I, II, and III are marked as *, **, and ***, respectively.

proportion with jaundice and peak ALT activity to be lower. The peak ALT activities observed in our SOTR cases are within the range reported in case series from western countries.^{31,33} The low activity is unlikely to be explained by the whitewash effect of steroids on liver transaminitis as all our non-SOTR immunosuppressed patients were on steroids and three of the eight SOTRs were not receiving steroids. It is also unlikely to be due to early diagnosis of hepatitis as the SOTRs were not subsequently treated with antivirals, which would curtail the ALT activity.

The trends in differences between SOTRs and non-SOTRs identified in this study were based on a small sample set. They require confirmation using a larger case series or a national notification database for hepatitis E.¹⁵ Future case definitions of hepatitis E would need to be based on PCR in addition to serology to generate accurate data.

Contrasting with HEV strains that are being reported from HEV-infected humans in east Asia, which belong predominantly to HEV-4,^{1,22} with the exception of Japan where both genotypes 3 and 4 co-circulate,¹⁶ all our cases were found infected by HEV-3, similar to what is being observed in the west.^{1,34} The disparity between our observations and those from other Asian countries may reflect differences in distribution of circulating HEV strains and genotypes. For SOTRs, differences in transplantation practices, posttransplant immunosuppression regimens, and lifestyle of transplant recipients (e.g., dietary avoidances), may be responsible.

In industrialized countries, including Singapore, the contribution of zoonotic HEV transmission to hepatitis E remains largely unknown.^{1,8} HEV infection and hepatitis E in Japan have been shown to be acquired from eating raw or undercooked meat and offal (including liver) of HEV-infected deer, boars, and pigs,³⁵ and in France from eating sausages prepared from raw pig liver.³⁶ In other countries, there is circumstantial evidence to implicate consumption of inadequately cooked porcine food products^{37,38} or of fecally contaminated shellfish.^{39–41} We have recently reported possible zoonotic HEV transmission via camel-derived food products.⁴²

Earlier studies attempting to define subgenotypes of HEV were based mainly on analysis of subgenomic regions. Subsequent analyses of whole-genomic sequences have shown that discrete boundaries may not distinguish subgenotypes consistently. Accordingly, the labeling of clades apparent within HEV-1, HEV-3, and HEV-4 sequence sets have been proposed.⁴³ With regard to HEV-3, there are three such clades into which its sequences aggregate. Sequences characterized from rabbits constitute one clade. Human and porcine sequences constitute the other two clades: one clade comprises subgenotypes a, b, c, h, i, and j (therefore is referred to as the 3abchij clade); the other comprises subtypes e, f, and g (hence, the 3efg clade).⁴⁴

Of the three lineages identified in this study, one lineage (Lineage I) is in the 3efg clade, and the other two lineages (Lineages II and III) belong to the 3abchij clade (Figure 1). Lineage I comprises sequences from Patients 5, 8, 9, and 15. For these 4 sequences, the mean distance among them, as calculated using the Kinura 2-parameter substitution model, was small, 0.094, contrasting with an average distance of 0.131 (range 0.123–0.137) to EU360977, the next closest sequence (Figure 1). As Patients 5 and 8 presented

no history of recent travel abroad, they were likely infected by HEV-3 that is circulating in Singapore. The other two patients (9 and 15) had recently traveled before their illness. However, the sequence of the strain that infected Patient 9, who traveled to Japan, was widely divergent from the next closest HEV-3 sequence from Japan (AB850879) (0.132). Thus, Patient 9 was likely infected by HEV-3 that was indigenous to Singapore, even though he had traveled to Japan. For the two Lineage II sequences from Patients 3 and 14, who had traveled to China, their distance was 0.021, compared with a distance of 0.08 to the next closest sequence, AF082843, which is from the United States, not China (Figure 1). Moreover, HEV genotype 3 sequences are rarely reported from China. From these considerations, Patients 3 and 14 likely acquired their infection from Singapore rather than China.

The TMRCA for the global HEV-3 sequences included in this study was circa 1853, a date which falls within previous estimates,²⁸ and attesting to the relatively recent introduction to human populations of HEV-3. Among the Singapore sequences, the TMRCA of Lineage I is the oldest (83 years ago). HEV strains in this cluster are inferred to have been introduced circa 1933, originating from Japan. About this time in history, massive emigration outflows from Japan were occurring: between 1869 and 1941, > 11,000 Japanese settled in Singapore and Malaya alone.⁴⁵ Movement into Singapore of people, animals, human-derived biologicals, and animal-derived food products could have introduced Lineage I strains into the human population. The more recent introduction of Lineage II strains, circa 1976, possibly from the United States, may reflect expanding globalization and growth of Singapore as an international trading, tourist, and commercial center, with consequent influx of HEV-infected people and importation of HEV-contaminated food products. It is speculated that subsequently, strains from these two lineages established endemicity, from which further transmissions ensued, with the transmissions becoming more frequent¹⁵ as Singapore gained affluence. Recent data from the United States suggest that the prevalence of HEV infection is correlated positively with affluence.^{46,47}

The HEV-3 strain whose sequence constituted Lineage III was characterized from Patient 16, who originated from Vietnam and also traveled regularly there before his stem cell transplant. Whether it was imported or indigenous to Singapore is uncertain. We note, however, that the sequence originated from the United States, its TMRCA being circa 1993.

Our TMRCA and phylogeographical studies were based on a 691-nt segment in the carboxyl half of the *ORF2* gene, which we had used in our previous study.²³ An even shorter segment (301 nt in length) used to estimate the evolutionary history of genotype 3 in Japan⁴⁸ generated estimates that were in good agreement with those using nearly full-genomic sequences.^{23,28} Accordingly, we are confident that the estimates obtained from using a longer segment would also agree with those from full-genomic sequences. However, over-representation of HEV-3 sequences from Japan⁴⁸ and the underrepresentation of corresponding sequences archived from Europe present sampling biases in our study and likely affect discrete trait modeling. This could affect the probable site of origin for the Group I sequences, as France and Germany have the second and

third highest location probabilities, 0.16 and 0.11, respectively, which albeit are low probabilities. To provide more accurate inferences of the emergence and origins of HEV strains, it would be appropriate to enroll study sequences more widely.

In summary, hepatitis E in Singapore bears features similar to what is observed in western countries, with respect to its detection among SOTRs and the predominant involvement of HEV-3. The HEV-3 strains belong to two main lineages: one lineage, in the 3efg clade, was introduced 83 years ago and probably originated from Japan; and the other, in the 3abchij clade, was introduced 40 years ago, from the United States. These strains and lineages likely underlie the current, rising endemicity of hepatitis E in Singapore.

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