involved. Although all P2Y receptors are activated by ATP, at $P2Y_1$, ADP is reported to be equipotent or more potent than ATP, and at $P2Y_2$, UTP and ATP are equipotent [46]. In addition, UTP levels are critical for efficient hepatitis C virus replication, and UTP depletion contributes to the development of liver injury [47, 48]. Whether ADP or UTP contributes to the effects of ALF-P on the growth of OCs is now under investigation.

Given the evidence on the importance of TNFα in the development of ALF and the negative correlation between ALF prognosis and TNFα levels, the present study suggests that a TNFα receptor signal is involved in the effects of ALF-P. Moreover, P2 receptor inhibition completely abolished the effects of TNFα on the proliferation and apoptosis of OCs, indicating that the TNFα pathway is P2 receptor-dependent. Our data showed no significant difference in plasma TNFα levels between the NC-P and the ALF-P samples. Although the plasma levels of other cytokines, including IL-5, IL-8, and IL-17, were significantly changed in the ALF-P compared with those in NC-P, because these interleukins do not interact with ATP or TNFα receptors, we can therefore predict that all the above cytokines do not contribute to the ALF-P-induced action of the TNFα receptors. On the other hand, several members of the TNF family besides TNFα serve as ligands of the TNF receptors [49]. There is another possibility that some unknown TNF ligands may contribute to the effects of ALF-P through TNF receptor signaling. These factors should thus be clarified in future investigations.

The components of normal human plasma have been reported [50, 51]. However, the exact nature of toxic molecules in the plasma during liver failure is unknown and the toxicity effects may vary among different organ systems [52]. In the present study, we focused on the effects of ATP, TNFα, and their related signals on the proliferation of oval cells based on the fact that both ATP and TNFα play essential roles in the development of fulminant hepatitis and in regulating the proliferation of hepatocytes. Although our data did not support that either ATP or TNFα should be the target molecule in the ALF plasma, the importance of $P2Y_2$ receptor crosstalk with the TNFα signaling pathway has been clearly addressed and subsequently will be valuable for our later investigations.

In conclusion, the present study demonstrated the specific involvement of JNK activation, the important roles of ATP receptor $P2Y_2$, and the crosstalk of $P2Y_2$ with TNFα receptor signaling in mediating the effects ALF-P on the regulation of OC growth. The data also suggested that targeting the JNK pathway could selectively inhibit the abnormal proliferation of OCs in ALF without affecting the growth of normal hepatocytes, which may be of clinical significance in the treatment of ALF.

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Conflict of interest The authors declare that they have no conflicts of interest.

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＜速　報＞

血中のA型肝炎ウイルス粒子には脂質膜エンベロープが存在する

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緒言：A型肝炎ウイルス（hepatitis A virus，HAV）は*Picornaviridae*科*Hepatovirus*属の“non-enveloped virus”である．主にfecal-oral transmissionの感染様式をとり，最初のウイルス分離も糞便材料からであったゆえ，糞便中ウイルスの方が形態学的あるいは物理化学的性状解析の優先対象となりやすく，血中ウイルスの方は等閑視されてきた．しかるに，夙に1975年にProvost等がマーモセット血中のHAV粒子比重のduality（1.15 g/m*l*と1.34 g/m*l*）を報告し[1)]，1985年にはLemon等が細胞培養系のHAVにも比重の軽い（1.14-1.18 g/m*l*）粒子と重い（1.32 g/m*l*）粒子の二種類があると報告し[2)]，時経て2010年には自治医大のTakahashi M等が，HAVと同じくfecal-oral transmissionの感染様式を取る“non-enveloped virus”の一種であるE型肝炎ウイルス（hepatitis E virus，HEV）が，血中では脂質膜を被った“enveloped virus”の形で存在していると報告するに及んで[3)]，HAVも血中ではHEVと同様に表面が脂質膜で覆われた存在形態を取っているのではないかとの推測が俄かに強まって来た．

方法：A型肝炎自施設例の凍結保存血清パネルからHAV RNA titerが高く且つ血清量の夛いものを選び解析対象とした．ウイルス粒子の比重はCsCl浮上密度勾配遠心で測定し，粒子径の推定は三段階のポアサイズ（80，50，30 nm）を有するCyclopore Membraneを用いて行なった．抗体（anti-HAV及びanti-CD59 antibodies）や各種レクチンとの結合能はmicro-affinity法[4)]により測定した．

結果：A型肝炎患者血清をCsCl浮上密度勾配遠心に掛けると，比重1.17 g/m*l*の分画にHAV RNAのピークが来た．この分画（1.17 g/m*l*）を蛋白分解酵素（Pronase™）＋脂肪族溶媒（chloroform）の組合せ，あるいは蛋白分解酵素（Pronase™）＋界面活性剤（Nonidet P-40™）の組合せで処理した後にCsCl密度勾配遠心に掛けると，いずれの場合にも，HAV RNAのピークが比重1.17 g/m*l*から比重1.31 g/m*l*へとシフトした．のみならず，未処理時のHAV RNAピーク分画（1.17 g/m*l*）には検出されなかったHAV抗原が，同上処理後のHAV RNAピーク分画（1.31 g/m*l*）には明瞭に検出された（Fig. 1）．また同上処理によりHAVの粒子径も50-80 nmから30-50 nmへと縮小した（data not shown）．また，未処理時のHAV RNAピーク分画（1.17 g/m*l*）にはanti-CD59 antibodies及び幾つかのレクチンとの結合能が明らかに存在したが，同上処理後のHAV RNAピーク分画（1.31 g/m*l*）ではこれら全てが検出不能となった（data not shown）．

考察：脂肪族溶媒あるいは界面活性剤で処理することにより，低比重から高比重へとシフトし，粒子径も有意に減少し，且つ処理前に隠れていたウイルス抗原が処理後には露出したという事実から，本研究の被検血清中に存在したHAV粒子の表面は脂質性皮膜で被覆されていたことが強く示唆された．本稿準備中に*Nature*に掲載されたFeng Z et alの論文[5)]では，彼等が命名するところの“eHAV”（enveloped HAVの意）の表面に存在する脂質性皮膜が電子顕微鏡下にも明瞭に捉えられている．彼等の使っている「宿主からハイジャックした脂質膜」というキャッチコピーは，我々の実験に於いても宿主細胞膜マーカーの一つであるCD59（所謂「脂質ラフト」の主要構成蛋白である）が検出されたことによりサポートされている．本来的にはnon-enveloped virusであるところのHAVやHEVが，このようにエンベロープを持った形で血中に出現して来ることの生物学的合目的性が奈辺に有るのかなど不明部分は多々残されているが，少なくとも，血中のHAV粒子が急性期流血中に同時存在するIgMクラスのHAV抗体により速やかに中和されてしまわないのは何故なのか？という積年の宿題には答が出た．また，糞便中HAVがnon-envelopedであるのは何故なのか？については，細胞破壊のない状況下で肝細胞から出て行くHAVは（行

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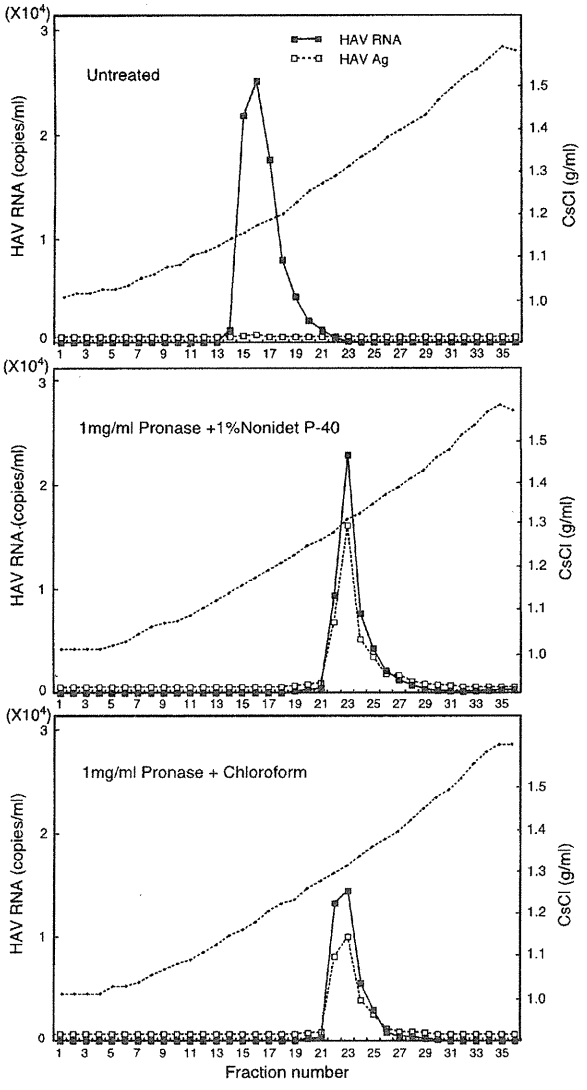

Fig. 1 A patient's serum that contained HAV RNA at 2.1×10^7 copies/m*l* was fractionated by CsCl density gradient ultracentrifugation (*top*). The HEV RNA-peak fraction was re-subjected to CsCl density gradient ultracentrifugation after treatment with protease/detergent (*middle*) or with protease/lipid solvent (*bottom*). Both treatments yielded in a shift of the HAV RNA peak from 1.17 to 1.31 g/m*l* and revelation of HAV antigen (HAV Ag).

先が血管方面であれ胆管方面であれ）須らく脂質膜を被った形で出て行くが，胆管に入ったヴィリオンは胆汁中の detergent と十二指腸内の protease の作用により表面の脂質膜を剥奪されてしまい，non-enveloped form に変容するからだと推測されるが，証拠は未だ無い．

結語：HAV は HEV と同様に，血中では脂質膜を被った“enveloped virus”の形で存在し，中和抗体による認識を擦り抜けていることが明らかになった．

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索引用語：A 型肝炎ウイルス，エンベロープ，脂質膜

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本論文内容に関連する著者の利益相反：なし

英文要旨

Circulating hepatitis A virus particles have a lipid-associated envelope

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We addressed a presumption that hepatitis A virus (HAV) particles in the blood circulation might be lipid-associated, and have significantly lower density and larger size than so far described in textbook. Sera from patients with acute hepatitis A served as materials. In CsCl density gradient ultracentrifugation, the serum-derived HAV particles banded initially at a density of 1.17 g/m*l*, but shifted to a heavier density of 1.31 g/m*l* when treated with protease (Pronase) plus lipid solvent (chloroform) or detergent (NP40). In parallel, the sizes of HAV particles also shifted from 50-80 nm to 30-50 nm in diameter by these treatments. The heavier HAV particles (1.31 g/m*l*) could bind to anti-HAV antibodies, while those with a low density (1.17 g/m*l*) could not. Conversely, the low density (1.17 g/m*l*) HAV particles could bind to anti-CD59 antibodies as well as some lectins, but the high density (1.31 g/m*l*) ones could not. These results suggested that HAV, despite taxonomically classified as a "nonenveloped" virus, exists in the blood of infected hosts as though being an "enveloped" virus, possessing a lipid membrane-like coat, which masks viral antigens from circulating antibodies.

Key words: hepatitis A virus, envelope, lipid membrane

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Review Article

Features of hepatitis E virus infection in humans and animals in Japan

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In Japan, hepatitis E had long been considered to be a rare liver disease which can be accidentally imported from endemic countries in Asia and Africa, where the sanitation conditions are suboptimal. However, since the identification of the first autochthonous hepatitis E case and hepatitis E viremic domestic pigs in Japan in 2001, our understanding of hepatitis E virus (HEV) infection in this country has been changing markedly. This has largely been due to the development of serological and gene-based diagnostic assays, the accumulation of molecular epidemiological findings on HEV infection in humans and animals (as potential reservoirs for HEV in humans) and the recognition of the importance of zoonotic food-borne and other routes of transmission of HEV, including blood-borne transmission. Although it is now evident that autochthonous hepatitis E in Japan is far more common than was previously thought, clinical and subclinical HEV infections indigenous to Japan remain underdiagnosed and their prevalence is still underestimated due to the presence of unknown transmission routes and a low awareness of the infection status by many physicians in Japan. This review focuses on the features of HEV infection in humans and animals, as definitive or potential reservoirs for HEV, in Japan, and updates the current knowledge on the routes of transmission, including zoonotic routes, which are important for the maintenance and spread of HEV in Japan.

Key words: hepatitis E, hepatitis E virus, genotype, zoonosis

INTRODUCTION

HEPATITIS E IS a form of acute hepatitis, which is caused by infection with hepatitis E virus (HEV), rarely leading to fulminant hepatitis with a high mortality rate. HEV principally replicates in the liver, is shed into the intestinal tract via the bile duct and is subsequently excreted into the feces. Therefore, the infection is transmitted primarily through the fecal–oral route, and the disease is highly prevalent in developing countries in Asia, Africa and Central America, where sanitation conditions are suboptimal.[1] Until very recently, HEV was regarded to be a rare "imported hepatitis" in industrialized countries, including the USA, European countries and Japan, and has consequently not attracted much attention in terms of research. However, the circumstances surrounding this disease have been very different since 1997. It has since become evident that indigenous HEV strains whose genotypes are different from those in endemic countries are circulating in industrialized countries, and that HEV infection is implicated in at least some cases of sporadic acute hepatitis or fulminant hepatitis whose etiology had been regarded to be unknown.[2–10] Furthermore, it has been demonstrated that HEV is the only zoonotic virus among the five known hepatitis viruses,[11–15] and that sporadic acute hepatitis E can occur through consumption of meat/viscera from domestic pigs or wild animals (boars and deer), which serve as reservoirs for HEV infection in humans.[16–20]

Hepatitis E virus is a non-enveloped, small, spherical virus with a diameter of 27–34 nm (mean, 30), and is classified as a member of the genus *Hepevirus* of the family Hepeviridae.[21] The genome of HEV is a single-stranded, positive sense RNA composed of 7.2 kilobases (kb), and possesses a short 5′-untranslated region

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(UTR), followed by three open reading frames (ORF: ORF1, ORF2 and ORF3) and then a short 3′-UTR.[22] ORF1 encodes non-structural proteins involved in viral replication and viral protein processing, while ORF2 codes for a 660-amino-acid (a.a.) capsid protein and ORF3 encodes a small protein of 113–114 a.a. that is required for virion egress from infected cells and is associated with numerous cellular pathways.[23–26] There are four recognized genotypes of HEV that infect humans.[27] Genotype 1 is responsible for the majority of HEV infections in developing countries in Asia and Africa; genotype 2 consists of strains detected not only in Mexico, but also in African countries; genotype 3 is widely distributed throughout the world; and genotype 4 is distributed mainly in Asian countries, including China, Indonesia, Taiwan, Vietnam and Japan.[28,29] Genotype 1 and 2 infections have been identified exclusively in humans and are responsible for water-borne epidemics, while genotype 3 and 4 viruses have been isolated from humans as well as pigs, wild boars, deer, mongooses and rabbits, raising public health concerns about zoonotic infection through direct contact with infected animals, or more likely, through the consumption of contaminated animal meat and viscera.[13,30–32]

Hepatitis E virus infection is generally a self-limited transient infection, and HEV is eliminated by the immune response of the host. Therefore, acute hepatitis E does not usually require antiviral therapy, although some patients may require treatment of symptoms. However, chronic HEV infection has recently been documented in immunocompromised solid-organ transplant recipients, HIV-infected patients and hematological patients receiving chemotherapy, and has been reported to progress rapidly to liver cirrhosis.[33–36] Treatment options for patients with chronic hepatitis E include reduction of immunosuppression[37] and administration of pegylated interferon-α or ribavirin.[38–41] Research on the treatment or prophylaxis of hepatitis E is an important issue in public health at the global level.

This article reviews the features of HEV infections in humans and animals in Japan, where hepatitis E has been a topic of interest since the independent identification of a hepatitis patient infected with an autochthonous genotype 3 HEV strain (JRA1) who had no history of traveling abroad, and evidence of HEV-infected domestic pigs in Japan in 2001.[15,42] This interest prompted many researchers in Japan to promote research on the diagnosis and epidemiology of HEV infections, and to clarify the importance of zoonosis in the maintenance and spread of HEV in the community.[9,10,13,16,17,29,43]

HEV INFECTION IN HUMANS

HEV infection in foreign countries and in foreigners living in Japan

THE PREVALENCE OF HEV infection is considered to be related to socioeconomic conditions in the country, although the geographic prevalence of antibodies against HEV (anti-HEV) is worldwide.[44,45] High prevalence is common in developing countries where large epidemics or outbreaks have occurred, while low prevalence is common in industrialized countries where sporadic infection has been occurring. Of interest, it has been reported that the positivity for HEV antibodies was 47.7% (143/300) in indigenous Chinese, 50.7% (152/300) in Korean living in Northeastern China, 34% (102/300) in indigenous Korean living in South Korea, 14.3% (43/300) in Koreans living in Japan and 6.0% (18/300) in indigenous Japanese,[46] suggesting that the prevalence of HEV infection may be associated with the living place where the sanitary conditions, route of transmission (water-borne or food-borne) and pattern of HEV infection (large or small outbreaks or sporadic infection) are different.

HEV infection in the general population of Japan

According to a nationwide survey of HEV infection in the general population of Japan, which was conducted for 22 027 individuals (9686 males and 12 341 females; age, mean ± standard deviation [SD], 56.8 ± 16.7 years; range, 20–108) who lived in 30 prefectures in Japan during 2002–2007, 1167 individuals (5.3%) were positive for the anti-HEV immunoglobulin (Ig)G class (anti-HEV IgG), including 753 males (7.8%) and 414 females (3.4%), with the difference between sexes being statistically significant ($P < 0.0001$) (Fig. 1).[47] The reason for the significant sex difference remains unknown. However, this trend could be linked to the behavior of each individual, with the higher frequency of alimentary or occupational exposure among males, because consumers of raw or undercooked meat or viscera of animals were frequent among male patients with hepatitis E,[16] and anti-HEV antibodies were prevalent among hunters.[48] Host factors are also probably implicated, although they are still unknown. Our preliminary unpublished observation indicated that children and young adults aged less than 20 years in Japan were less frequently infected with HEV and that the prevalence of anti-HEV IgG is less than 1% in this population. Based on the population statistics available from the Portal Site of Official Statistics of Japan (http://www.e-

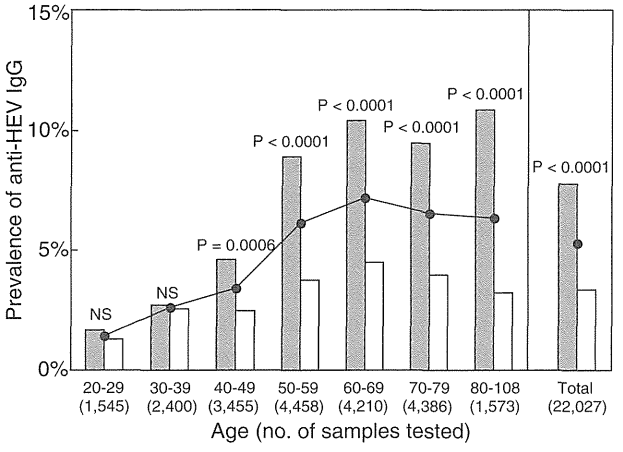

Figure 1 Age-dependent prevalence of anti-hepatitis E virus (HEV) immunoglobulin (Ig)G among 22 027 apparently healthy individuals. Modified from reference.[47] ▩, male; □, female; —●—, total.

stat.go.jp) and the age- and sex-dependent prevalence of anti-HEV IgG, approximately 5 million people are estimated to have had a past HEV infection. The prevalence of anti-HEV IgG generally increased with age and was significantly higher among individuals aged 50 years or older than among those aged less than 50 years (6.6% vs 2.7%). Based on the nearly linear increase in the anti-HEV IgG prevalence rate from the age group of 20–29 years to the age group of 60–69 years in Japan, the annual incidence of HEV infection was calculated to be 0.15% (males, 0.22%; females, 0.08%), which is close to that of 0.14% in hemodialysis patients, with an appearance rate of anti-HEV IgG of 1.07% (4/374) occurring during the average observation period of 7.7 years[49] and that of 0.09% in medical employees, with an emergence rate of anti-HEV IgG of 0.77% (2/260) during a mean observation period of 8.7 years.[50] Consequently, referring to the population statistics, the annual number of HEV infections in Japan is estimated to be approximately 150 000 (108 000 males; 42 000 females).

Three of the 22 027 individuals assessed as part of the nationwide survey were positive for HEV RNA, despite being negative for anti-HEV IgG, IgM and IgA, likely due to the collection of blood samples during the window phase of HEV infection, suggesting that approximately one in 7300 healthy people has an ongoing HEV infection at any particular time point. The HEV strains isolated from three viremic individuals belonged to genotype 3, similar to the indigenous HEV strains previously identified in Japan.[47]

The prevalence of anti-HEV IgG was significantly higher among individuals living in the northern part of Japan (Hokkaido, Tohoku, Kanto and Chubu) than among those living in the southern part of Japan (Kinki, Chugoku, Shikoku and Kyushu) (6.7% vs 3.2%, $P < 0.0001$). Notably, the prevalence of anti-HEV IgG was significantly higher among males than among females in all eight regions of Japan (Fig. 2). All but one individual with HEV RNA or anti-HEV IgM and/or anti-HEV IgA lived in the northern part of Japan. In other words, the prevalence of HEV RNA or anti-HEV IgM and/or anti-HEV IgA was also significantly higher among individuals living in the northern part of Japan than among those living in the southern part of Japan (15/13 182 [0.11%] vs 1/8845 [0.01%], $P = 0.0056$]. Similar regional differences in the anti-HEV IgG prevalence rate also have been found in blood donors in Japan.[51] Of interest, when the prevalence rate of anti-HEV IgG was compared with the number of pigs raised on swine farms in each of the 30 prefectures studied (http://www.maff.go.jp/j/tokei/kouhyou/tikusan/index.html), a positive correlation was observed (correlation coefficient = 0.5104). The prevalence rate of anti-HEV IgG also correlated closely with the monthly expenditure for pork in each prefecture (http://www2.ttcn.ne.jp/~honkawa/7238.html) (correlation coefficient = 0.5102). These observations may explain the regional differences in the prevalence of HEV infection

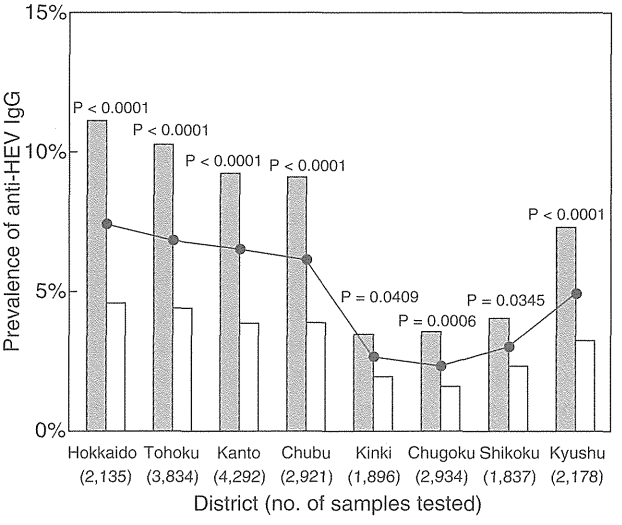

Figure 2 Geographic region-dependent anti-hepatitis E virus (HEV) immunoglobulin (Ig)G distribution among 22 027 apparently healthy individuals. Modified from reference.[47] ▩, male; □, female; —●—, total.

in Japan, and support the importance of pigs as reservoirs for HEV infection in humans.

Characteristics of autochthonous hepatitis E in Japan

In 2003, we summarized the clinical courses and symptoms of domestic HEV infections in Japan,[10] by analyzing 46 Japanese patients who were diagnosed with hepatitis E in our laboratory based on the presence of both anti-HEV IgM and HEV RNA in their sera that had been obtained at admission, including 11 of 87 (13%) patients who had previously been diagnosed with sporadic acute hepatitis of non-ABC etiology[8] and three of 18 (17%) patients who had received a diagnosis of fulminant hepatitis of unknown etiology.[9] Until the end of 2012, we had an opportunity to diagnose hepatitis E in 153 additional patients who had neither history of travel to endemic areas nor contact with travelers abroad or foreigners within 3 months before disease onset. In this review, we therefore summarize the characteristics of 199 domestic hepatitis E cases in Japan, in comparison to eight patients with imported hepatitis E (Table 1).

To diagnose hepatitis E, serum samples were tested for the presence of anti-HEV IgG, IgM and IgA by an in-house enzyme-linked immunoassay (ELISA) with recombinant ORF2 protein,[52] as well as for HEV RNA by nested reverse transcription polymerase chain reaction (RT–PCR) with primers targeting the ORF2 region.[8] Among the 207 total patients with hepatitis E, all but one patient were exclusively positive for anti-HEV IgG, IgM and IgA, while the remaining patient was negative for anti-HEV IgM throughout the observation period, despite positivity for anti-HEV IgG and IgA.[53] Because, among the 2781 samples from subjects who were assumed not to have been recently infected with HEV as negative controls, the false-positive rate was significantly lower when the anti-HEV IgA assay than when the anti-HEV IgM was assay used (the estimated false-positive rates of the assays were 0.6 vs 0.1%; $P = 0.0139$: McNemar's χ^2-test),[52] an anti-HEV IgA assay system has been used for the serological diagnosis of acute hepatitis E in the clinical setting in Japan since it started to be covered by the government insurance program in October 2011. All 207 patients had detectable HEV RNA in each initial serum sample obtained 0–77 days (mean ± SD, 8.2 ± 8.4; median, 6.0) after the disease onset.

The clinical and epidemiological characteristics of domestic hepatitis E in the 199 patients, including a 38-year-old male who developed autochthonous hepatitis E in 1982,[54] are summarized as follows: (i) the patients were distributed all over Japan, but there was a wide variation in the geographical distribution of hepatitis E, with a higher prevalence in Hokkaido, accounting for one-third of the total infections, and in the northern part of mainland Honshu (Tohoku and Kanto); (ii) 159 (80%) patients were male; (iii) the age of the patients ranged 18–86 years, with a mean age of 56.8 years, and the patients aged 50 years or older accounted for approximately 70% of the total, contrasting with imported cases, who had a mean age of 37.9 years; (iv) 22 of the 199 patients (11%) had a

Table 1 Characteristics of patients with hepatitis E in Japan

Hepatitis	Region†	No. of cases	Male	Age (years)		No. of cases with a diagnosis of hepatitis E			No. of cases with HEV genotype			
				Mean ± SD	Range	AH	AHS	FH	1	3	4	3 + 4
Domestic	Hokkaido	65	53 (82%)	56.5 ± 13.1	25–86	56	7	2	0	14	51	0
	Tohoku	60	48 (80%)	56.5 ± 12.1	18–82	50	7	3	0	52	8	0
	Kanto	35	24 (69%)	57.3 ± 14.9	28–78	30	3	2	0	29	5	1
	Chubu	17	14 (82%)	52.7 ± 11.0	28–71	17	0	0	0	12	5	0
	Kinki	16	15 (94%)	59.1 ± 11.0	36–77	11	5	0	0	16	0	0
	Chugoku/Shikoku	2	1 (50%)	73.5 ± 12.0	65–82	2	0	0	0	2	0	0
	Kyushu/Okinawa	4	4 (100%)	62.0 ± 10.9	48–72	4	0	0	0	3	1	0
Subtotal		199	159 (80%)	56.8 ± 12.8	18–86	170	22	7	0	128	70	1
Imported		8	8 (100%)	37.9 ± 14.1	23–58	7	0	1	5	0	3	0
Total		207	167 (81%)	56.1 ± 13.3	18–86	177	22	8	5	128	73	1

†Located from north to south in Japan.
AH, acute hepatitis; AHS, a severe form of acute hepatitis with a lowest prothrombin time of <40%, but without hepatic encephalopathy; FH, fulminant hepatitis; HEV, hepatitis E virus; SD, standard deviation.

lowest prothrombin time of less than 40%, unaccompanied by hepatic encephalopathy, and were diagnosed with severe acute hepatitis, and seven other patients (4%) contracted fulminant hepatitis; (v) among the 199 patients with domestic hepatitis E, 128 patients (64%) had genotype 3 HEV, 70 patients (35%) had genotype 4 HEV, and the remaining patient was co-infected with genotype 3 and 4 viruses.[55] In contrast, among the eight patients with imported hepatitis E, five patients had genotype 1 HEV, due to infection in Bangladesh, India or Nepal,[56] while the remaining three patients had genotype 4 HEV, all of whom were presumed to have contracted HEV infection while traveling in China or Vietnam.[57] With regard to the significant sex difference, a similar demographic profile with the majority of clinical HEV infections being described in middle-aged and elderly men has also been reported in other countries including France, Germany, the UK and the USA.[48,58–60] As possible host risk factors important for clinical disease expression, excessive amounts of alcohol drinking and subclinical hepatic steatosis/fibrosis have been suggested.[61,62]

Since 2008, chronic cases of HEV infection have been reported in solid-organ transplant patients, HIV patients and hematological patients receiving chemotherapy in Europe and North America.[33–35,63] In Japan, the assessment of the prevalence and incidence of chronic HEV infection in these populations is now underway, although persistent infection of HEV transmitted by blood transfusion in a Japanese patient with T-cell lymphoma had been already reported in 2007.[64]

Clinical significance of the HEV genotype

Multiple HEV strains of genotype 3 or 4 have been isolated from Japanese patients with autochthonous hepatitis E (Fig. 3). In our previous study,[65] when compared with the seven patients with genotype 3 HEV, the 25 patients with genotype 4 HEV had a significantly higher peak alanine aminotransferase (ALT) level and a significantly lower level of lowest prothrombin activity, suggesting that the HEV genotype is one of the important risk factors associated with the disease severity. Notably, in Hokkaido, the majority of hepatitis E patients were infected with genotype 4 HEV, while approximately 90% of blood donors who were diagnosed as having ongoing HEV infection by a nucleic acid amplification test for HEV had genotype 3 HEV.[66] When the 202 patients infected with genotype 3 and/or 4 HEV (Table 1) were compared with 40 individuals with subclinical infection with genotype 3 or 4 HEV, including voluntary blood donors, apparently healthy persons who underwent health check-ups, and patients on hemodialysis,[47,49,50,67–69] genotype 4 HEV was significantly more prevalent in patients with clinical HEV infection than in individuals with subclinical HEV infection (74/202 [37%] vs 3/40 [8%], $P = 0.0006$: χ^2-test).

Fulminant hepatitis occurred significantly more frequently in patients infected with genotype 4 HEV than in those infected with genotype 3 HEV (6/74 [8.1%] vs 1/128 [0.8%], $P = 0.0191$: χ^2-test). In addition, among the 202 patients with clinical HEV infection, the peak ALT level and peak total bilirubin level were significantly higher in patients with genotype 4 HEV than in those with genotype 3 HEV ($P = 0.0026$ and $P < 0.0001$, respectively: Mann–Whitney *U*-test) (Table 2). When the HEV RNA titer in serum samples taken within 10 days after the disease onset between 99 patients infected with genotype 3 HEV and 55 patients with genotype 4 HEV was compared, it was found to be significantly higher in the serum samples from patients with genotype 4 HEV than in those from patients with genotype 3 HEV (median, 3.5×10^5 copies/mL vs 7.3×10^4 copies/mL, $P = 0.0130$: Mann–Whitney *U*-test). These findings further support our previous observation that HEV of genotype 4 is associated with more aggressive hepatitis than genotype 3. A high replication activity of genotype 4 HEV was reproduced in a cell culture system for the HE-JF5/15F strain of this genotype,[70] and this model is expected to shed light on the role of viral factors in the development of fulminant hepatitis E.[71]

HEV INFECTION IN ANIMALS

HEV infection in domestic pigs in Japan

IN 1997, MENG *et al.*[12] first reported the discovery of HEV in domestic pigs (*Sus scrofa domesticus*) in the USA. In Japan, the circulation of swine HEV strains on swine farms was first recognized in 2001.[15] However, a later study indicated that a presumably Japan-indigenous swine HEV isolate had been circulating in Japan in 1990.[72] Japan-indigenous HEV strains of genotype 3 have been subdivided into three lineages, including New World strains (subgenotype 3a), Japanese strains (3b) and European strains (3e).[28] The molecular tracing of HEV in Japan suggested that the oldest lineage, 3b, appeared around 1929, while lineages 3a and 3e appeared around 1960, coinciding with the increase of large-race pig importation from Europe and the USA.[73] The indigenization and spread of HEV in Japan are likely associated with the popularization of eating pork.

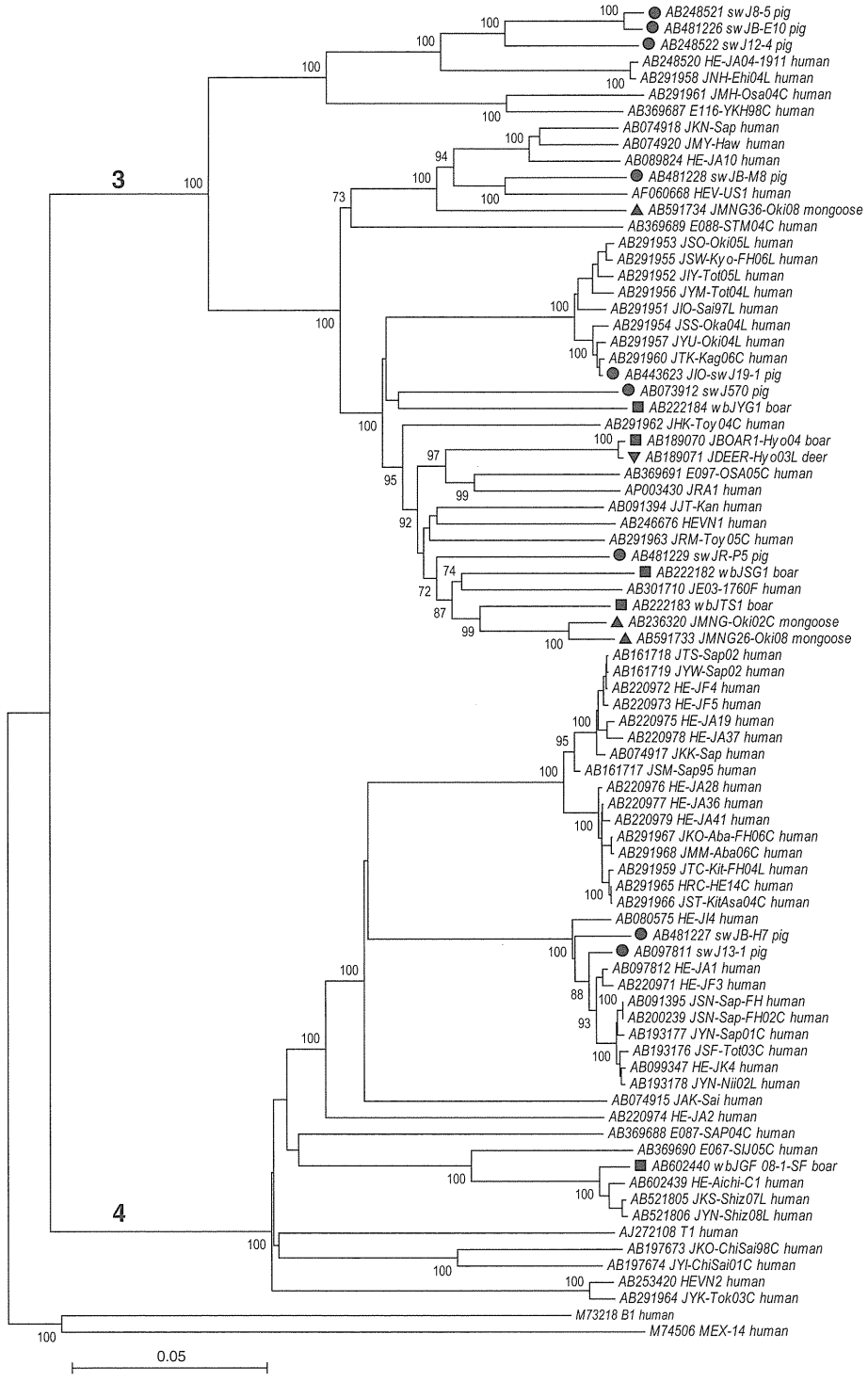

Figure 3 Phylogenetic tree constructed by the neighbor-joining method based on the entire or near-entire genomic sequence of 79 hepatitis E virus (HEV) strains of genotypes 3 and 4 isolated in Japan, including those obtained from pigs (●), wild boars (■), a deer (▼) and mongooses (▲), using prototype genotype 1 and 2 human HEV isolates as outgroups. The bootstrap values are indicated for the major nodes as a percentage obtained from 1000 resamplings of the data.

Table 2 Comparison of various features between patients with genotype 3 HEV and those with genotype 4 HEV

Feature	Patients with HEV		*P*-value
	Genotype 3 ($n = 128$)	Genotype 4 ($n = 74$)	
Age (mean ± SD) years	56.4 ± 13.0	57.5 ± 12.3	NS
Male	98 (76.6%)	64 (86.5%)	NS
Fulminant hepatitis	1 (0.8%)	6 (8.1%)	**0.0191**
Peak ALT (mean ± SD) IU/L	2310 ± 1800	2979 ± 1887	**0.0026**
Peak AST (mean ± SD) IU/L	2146 ± 2226	2503 ± 2049	NS
Peak total bilirubin (mean ± SD) mg/dL	6.9 ± 8.2	10.2 ± 7.9	**<0.0001**
Lowest prothrombin time			
<60%	25 (19.5%)	26 (35.1%)	**0.0139**
<40%	16 (12.5%)	11 (14.9%)	NS
HEV RNA (mean ± SD) copies/ml			
0–30 days after disease onset† ($n = 199$)	$2.0 \times 10^6 \pm 1.1 \times 10^7$	$2.6 \times 10^6 \pm 1.1 \times 10^7$	**0.0113**
0–10 days after disease onset† ($n = 154$)	$1.3 \times 10^6 \pm 3.9 \times 10^6$	$3.4 \times 10^6 \pm 1.3 \times 10^7$	**0.0130**

†Only 199/154 patients whose first serum sample was obtained during the first 30/10 days after disease onset were compared.
Bolding indicates significance.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; HEV, hepatitis E virus; SD, standard deviation.

To clarify the present status of HEV infection among domestic pigs in Japan, serum samples obtained from 3925 pigs aged 1–6 months on 117 farms in 21 prefectures, from Hokkaido to Okinawa, in Japan were studied for the presence of anti-HEV IgG by an in-house ELISA and HEV RNA by nested RT-PCR with ORF2 primers.[13,74] These nationwide studies revealed that antibody positive pigs were present in all 21 prefectures and 109 of the 117 (93%) farms studied, indicating the spread of HEV infection in pigs throughout Japan. The prevalence of anti-HEV IgG was 57% in total, and increased with age, reaching 84% in 6-month-old pigs (Table 3). Swine HEV generally infects pigs of 2–4 months of age. The titer of anti-HEV IgG also increased with age, peaked at 4 months of age, and then decreased, reflecting a transient infection of swine HEV during an early growing stage of the piglets. The positive rate of HEV RNA in the serum was highest in the 3-month-old pigs (14% or 145/1060), while none of the 386 pigs aged 6 months old tested had detectable HEV RNA. The swine HEV strains in Japan were segregated into genotype 3 or 4.[13,74]

Table 3 Age-dependent prevalence of anti-HEV IgG and HEV RNA in domestic pigs in Japan

Age (months)	Anti-HEV IgG positive	HEV RNA positive
1	21/218 (10%)	0/218
2	71/698 (10%)	11/378 (3%)
3	509/1060 (48%)	145/1060 (14%)
4	583/680 (86%)	34/360 (9%)
5	732/883 (83%)	2/383 (1%)
6	326/386 (84%)	0/386
Total	2242/3925 (57%)	192/2785 (7%)†

†Among 192 viremic pigs, 180 pigs were infected with HEV of genotype 3, and the remaining 12 pigs had HEV of genotype 4.
HEV, hepatitis E virus; Ig, immunoglobulin.

Considering food safety, it is fortunate that HEV viremia was not detected in any of the 6-month-old pigs ready for sale.[13,74] However, the identification of HEV in the pig liver sold as food in grocery stores (1.9% or 7/363 packages) suggest that raw or inadequately cooked liver, as well as meat and intestines from pigs, are associated with a risk of transmitting HEV to humans.[16] Of note, one swine HEV isolate of genotype 4 from a packaged pig liver had 100% identity with a HEV isolate (HE-JA18) obtained from a patient who developed sporadic acute hepatitis E after consuming pig liver, and two other swine HEV isolates of genotype 3 from packaged pig liver had 98.5–100% identity with a HEV isolate (HE-JA4) recovered from a patient who had a habit of eating pig meat/viscera.[16] Three cases of acute or fulminant E caused by ingestion of pork and pig entrails at a barbecue in a restaurant in Hokkaido, who were infected with HEV sharing 99.9–100% nucleotide sequence identity, have recently been reported.[75] In our cell culture systems for HEV using PLC/PRF/5 cells (hepatocellular carcinoma) and A549 cells (lung cancer) as host cells,[76] the HEV in homogenates of pig liver sold as food propagated efficiently and produced infectious progeny viruses (Fig. 4a),[77] demonstrating the infectivity

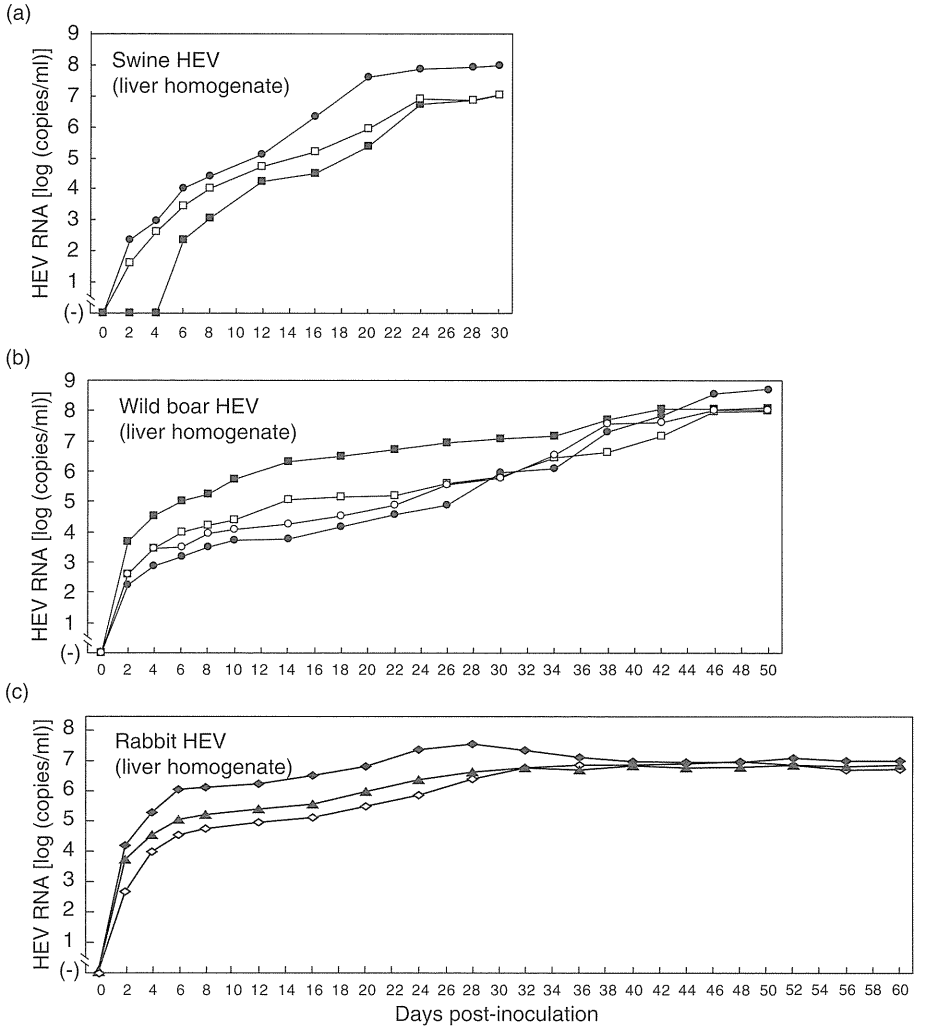

Figure 4 Quantitation of the hepatitis E virus (HEV) RNA in the culture supernatant of A549 cells inoculated with homogenate samples of liver tissues from (a) pigs, (b) wild boars and (c) rabbits. Modified from references.[77,78] (a) ■, swJL82; □, swJL97; ●, swJL98. (b) ■, wbJYG_05; □, wbJSO_05-2; ●, wbJGF_08-1; ○, wbJNR_10. (c) ◆, rbIM223L; ▲, rbIM227L; ◇, rbIM214L.

of HEV in pig liver that was for sale for human consumption. Taken together, although there is currently no direct evidence to prove HEV infection from pigs to humans, it is beyond doubt that pigs are the most important animal reservoir for HEV infection in humans in Japan, especially in Hokkaido, where the highest number of patients with hepatitis E have been reported, most likely due to the consumption of pig meat/viscera (Table 4).

HEV infection in wild boars in Japan

Wild boars (*Sus scrofa*) are indigenous to many countries worldwide, including Japan, posing ecological and infectious disease concerns. In some countries including Japan, recreational hunting of wild boars and the consumption of boar meat provides an increased risk for the transmission of various pathogens, such as HEV, from wild boars to humans. The prevalence of HEV infection among wild boars in Japan has been investigated by many researchers, and the findings are summarized in Table 5.[32,79–84] The HEV seropositivity in wild boars varied from 4.5% to 34.3%, and the HEV RNA detection rate ranged 1.1–13.3% in wild boars from different geographical regions in Japan.

In 2003, Matsuda *et al.*[18] reported, for the first time, two patients who developed a severe HEV infection after

Table 4 Likely sources of heptitis E virus infection in patients with autochthonous hepatitis E in Japan

District	No. of patients	Ingestion before the disease onset (within 2 months)				Contact with pigs and/or meat/viscera of pigs§	Unknown
		Pig liver†‡	Meat/viscera from wild boar‡	Meat/viscera from deer‡	Uncooked shellfish		
Hokkaido	65	45 (69.2%)	0	1 (1.5%)	4 (6.2%)	0	17 (26.2%)
Tohoku	60	9 (15.0%)	3 (5.0%)	1 (1.7%)	3 (5.0%)	4 (6.7%)	42 (70.0%)
Kanto	35	13 (37.1%)	0	2 (5.7%)	4 (11.4%)	0	19 (54.3%)
Chubu	17	1 (5.9%)	5 (29.4%)	1 (5.9%)	0	0	11 (64.7%)
Kinki and others¶	22	7 (31.8%)	4 (18.2%)	1 (4.5%)	2 (9.1%)	0	12 (54.5%)
Total	199	75 (37.7%)	12 (6.0%)	6 (3.0%)	13 (6.5%)	4 (2.0%)	101 (50.8%)

†Includes the colon and intestine from pigs.
‡Cooked, undercooked or uncooked.
§Includes patients who were pig farmers or who handled raw meat/viscera of pigs without gloves.
¶Includes Chugoku, Shikoku, Kyushu and Okinawa.

consuming raw liver from a wild boar. Later, Shimizu *et al.*[85] described four cases of acute hepatitis E in Aichi prefecture that occurred after the ingestion of boar meat. The HEV strains isolated from these patients that belonged to genotype 4, formed a single cluster, and were 98.8–99.7% identical to those recovered from wild boars captured in the same prefecture. Zoonotic food-borne transmission of HEV from wild boars to humans has been demonstrated by analyzing a case of hepatitis E caused by ingestion of boar meat, with the HEV strain sharing 99.95% nucleotide sequence identity with that in the leftover boar meat the patient had eaten.[19] Upon inoculation of A549 or PLC/PRF/5 cells with HEV in liver homogenates at 9.8×10^5 copies per well or more (six-well plate), the boar HEV multiplied efficiently (Fig. 4b) and produced infectious progeny viruses.[77] These findings indicate that wild boars are another important reservoir for HEV in humans.

Most strains of HEV recovered from wild boars worldwide belong to genotype 3.[30] However, in Japan, boar HEV strains of not only genotype 3, but also genotype 4, have been detected. In addition, two novel HEV strains (JBOAR135-Shiz09 and wbJOY_06) belonging to unrecognized genotypes (provisionally designated as genotypes 5 and 6, respectively) have been recovered from wild boars in Japan.[86–88] It remains unknown whether these novel HEV strains can be transmitted to humans, and are also part of the reservoir for HEV.

Table 5 Prevalence of HEV infection among wild boars in Japan

District (prefecture)	Anti-HEV IgG positive	HEV RNA positive	HEV genotype				Reference
			3	4	5	6	
Gunma	4/89 (4.5%)	1/89 (1.1%)	1	0	0	0	Sakano *et al.*[79]
Shizuoka	48/140 (34.3%)	5/140 (3.6%)	1	3	1†	0	Terada *et al.*[83]
Aichi	NA	11/91 (12.1%)	0	11	0	0	Ito *et al.*[80]
Hyogo	70/417 (16.8%)	16/436 (3.7%)	16	0	0	0	Kitajima *et al.*[84]
Ehime	100/392 (25.5%)	12/392 (3.1%)	10‡	0	0	0	Michitaka *et al.*[81]
Okinawa	NA	2/15 (13.3%)	0	2	0	0	Nakamura *et al.*[82]
Nationwide§	41/507 (8.1%)	19/578 (3.3%)	14	4	0	1¶	Sato *et al.*[32]
Total	263/1545 (17.0%)	66/1741 (3.8%)	42	20	1	1	

†The JBOAR135-Shiz09 isolate (AB573435) was provisionally classified into a novel genotype (genotype 5).
‡Ten out of 12 HEV RNA positive samples were subjected to HEV genotyping.
§Includes 25 prefectures in Japan.
¶The wbJOY_06 isolate (AB602441) was provisionally classified into a novel genotype (genotype 6).
HEV, hepatitis E virus; Ig, immunoglobulin; NA, not applicable.

HEV infection in wild deer in Japan

In 2003, Tei *et al.*[17] reported four patients who became infected with HEV after eating venison. Upon testing, a leftover portion of the meat, which had been kept frozen in anticipation of eating it in the future, was found to be positive for HEV RNA, and had a nucleotide sequence identical to those from the patients, providing direct evidence that HEV infection was a zoonosis. A full-length sequence of HEV genomes amplified from the venison shared 99.7% nucleotide sequence identity to a virus recovered from a wild boar hunted in the same forest where the implicated deer was captured, and to those from four patients who contracted hepatitis E after eating raw meat from the deer, suggesting an interspecies HEV transmission between wild boars and deer in the wild.[89] Genotype 3 strains of HEV have also been identified from deer in Hungary.[90] Thus, the deer HEV is considered to be zoonotic. However, the prevalence of anti-HEV IgG in deer was low, at 1.7% (2/117)[91] or 2.6% (25/976),[92] and no deer HEV strain, except for that recovered from the above-mentioned venison, has been identified in Japan.[93] Therefore, it has been suggested that deer may not play a major role as a HEV reservoir.[92]

HEV infection in wild mongooses in Okinawa, Japan

Hepatitis E virus infection has been reported in wild mongooses on Okinawa Island, Japan, with the HEV seroprevalence reported to be 8.3–21%.[94,95] The full-length genomic sequence of a strain (JMNG-Oki02C) of HEV recovered from a mongoose was determined, and was shown to be classifiable into genotype 3, with a close relationship to a genotype 3 swine HEV from Japan.[95] A recent study indicated that, among 209 wild mongooses tested in Okinawa, six (2.9%) were positive for HEV RNA, and the mongoose HEV strains of genotype 3 were segregated into two distinct lineages, 3a and 3b, both of which are also prevalent in humans and domestic pigs in Japan. Although the ability of the mongoose HEV to infect across species remains unknown, these observations emphasize the possibility that the mongoose may be a reservoir animal for HEV in Okinawa.[96]

HEV infection in other animals

Other than the domestic pigs, wild boars, deer and mongooses described above, antibodies against HEV have been detected in numerous animal species, including dogs, cats, sheep, goats, horses, cattle, bison and rats; and HEV or HEV-like strains have been genetically identified from chickens and rabbits, and recently from rats, bats, ferrets and fish (trout), which have further broadened our understanding of the host range and diversity of HEV.[30,97–99]

Recently, novel HEV strains that are close to genotype 3 HEV have been identified from rabbits in China, the USA and France.[78,100–105] The transmissibility of rabbit HEV to cynomolgus macaques,[106] the isolation of a HEV strain from a hepatitis E patient in France that formed a cluster with rabbit HEV strains,[107] and the successful propagation of rabbit HEV strains in human cultured cells (PLC/PRF/5 and A549 cells) (Fig. 4c),[78] suggest that rabbits are another likely source of human HEV infection. In Japan, infection of domestic and wild rabbits with HEV has not yet been reported.

Rats have long been suspected to be a potential reservoir for HEV. Antibodies against HEV have been detected in various species of rats, including Norway (*Rattus norvegicus*) and black (*Rattus rattus*) rats.[108–110] Until recently, the source of anti-HEV seropositivity in rats could not be identified. However, in 2010, Johne *et al.*[111] identified a novel HEV sequence from rats in Germany, which shared only 53–55% sequence identity with human HEV. Various rat HEV strains have now been identified in Germany, the USA, Vietnam and Indonesia, but not in Japan.[111–114] It remains to be determined if the rat HEV can cross the species barrier and infect humans or other animal species.

Recently, HEV-like viruses, forming novel phylogenetic clades in the family Hepeviridae (Fig. 5) have been identified from ferrets in the Netherlands,[115] from

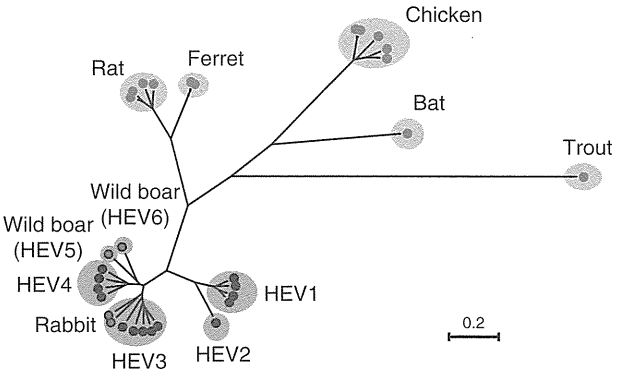

Figure 5 Phylogenetic tree constructed by the neighbor-joining method based on the entire genomic sequence of human-related hepatitis E virus (HEV) strains of genotypes 1–6 including those obtained from rabbits and wild boars and HEV-like strains including those obtained from rats, ferrets, a bat, chickens and trout.

African, Central American and European bats,[116] and from cutthroat trout in the USA.[99] So far, no clinical disease has been reported to be associated with these HEV-like viruses in humans and animals.

In addition, anti-HEV IgG antibodies have been detected by ELISA using the human HEV-derived ORF2 protein as antigen probe in various animals including cattle,[117–120] horses,[121,122] sheep,[118,123,124] goats,[120,123] dogs[117,121,125] and cats.[126,127] However, it remains unknown whether these animals are infected with human-related HEV or animal-specific HEV-like viruses.

A HEV-like virus, named avian HEV, has also been identified in chickens with or without hepatitis-splenomegaly syndrome in Australia, European countries, the USA, China and Korea.[128–132] Avian HEV in chickens shares only approximately 50% nucleotide sequence identity across the full-length genome with human and swine HEV. No data on the circulation of avian HEV in chickens in Japan are available so far.

POSSIBLE MODE OF HEV TRANSMISSION

SEVERAL CASES OF post-transfusion clinical or subclinical HEV infection have been reported in Japan,[64,133,134] including a case of transfusion-transmitted HEV infection in 1979 which was identified through a retrospective study among hemodialysis patients.[49] Therefore, the potential risk of transfusion-associated hepatitis E is not negligible. As mentioned above, sporadic cases or clusters of zoonotic food-borne HEV infection have been reported from various parts of Japan, particularly from Hokkaido where hepatitis E is endemic. Among 199 domestic hepatitis E cases in Japan, a food source was identified in 94 cases (47%) in total, including 48 cases (74%) in Hokkaido, with the leading cause being the consumption of uncooked or undercooked liver/colon/intestine from pigs (Table 4). It is therefore clear that the main route of HEV transmission is zoonotic food-borne transmission in Japan. Japan-indigenous genotype 3 HEV was detected in two of 32 packages of a bivalves called Yamato-Shijimi (*Corbicula japonica*) obtained from Japanese rivers, indicating that HEV also contaminates the river water in Japan.[135] Vietnam-indigenous genotype 4 HEV was obtained from a Japanese patient with imported hepatitis E who had consumed uncooked shellfish (a bivalve) while traveling in Vietnam.[57] It has also been reported that 8.7% of raw oysters collected from the coastal regions in Korea tested positive for HEV belonging to genotype 3.[136] Ishida *et al.*[93] reported that genotype 3 HEV was detected in a sewage sample and a seawater sample in Japan. In other reports, the isolation of HEV from sewage and river water raised the possibility of the contamination of shellfish by infectious HEV.[137,138] Therefore, river water contaminated with swine feces or incompletely sanitized sewage may prove to be the principal source of HEV contamination in shellfish. At present, the route of HEV transmission is unknown for nearly half of autochthonous hepatitis E cases, and the possible source of infection is considered to differ by geographic region in Japan (Table 4). Although six (3.0%) of the 199 patients with domestic hepatitis E reported ingestion of venison before the disease onset, the low prevalence of HEV infection among wild deer may suggest the necessity of considering other unrecognized infectious source(s). Further efforts to clarify the sources and routes of infection are needed to improve the control of infection of this zoonotic, food-borne hepatitis virus in Japan.

CONCLUSIONS

HEPATITIS E HAD been considered to be a travel-associated, acute, limiting liver disease that rarely progresses to fulminant hepatic failure in Japan. However, it became evident that HEV infection can also be acquired in Japan, as a zoonotic disease, with several species of animals, including pigs and wild boars, serving as reservoirs for HEV in humans. Since the recognition of the presence of a domestic hepatitis E case and HEV-viremic domestic pigs in 2001, serological and PCR-based assay systems for HEV infection have been developed, and knowledge on the genomic diversity of HEV strains in humans and animals has been broadened. In addition, sporadic cases and clusters of autochthonous hepatitis E in many parts of Japan have been accumulated, contributing to a better understanding of the pathogenesis of HEV infection. Furthermore, a serological test for hepatitis E, which is covered by the government insurance program, has been included in the strategy for the diagnosing acute hepatitis since October 2011 in Japan, and should be used to evaluate all patients with increased levels of liver transaminases. Because chronic hepatitis E has been observed in organ transplant recipients and HIV-infected patients in European countries and North America, it is necessary to test immunocompromised individuals with elevated liver enzymes for HEV RNA, and to elucidate their infection status in Japan, because such populations are also likely affected in our country. The animal reservoirs for HEV and the route/source of transmission are not fully understood. When the apparent zoonotic nature and

chronicity of HEV are taken into consideration, control of this virus seems to be difficult. Continued efforts to develop more accurate diagnostic systems by serological and molecular approaches, effective antiviral drugs for HEV and preventive vaccines using native HEV virions are warranted.

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