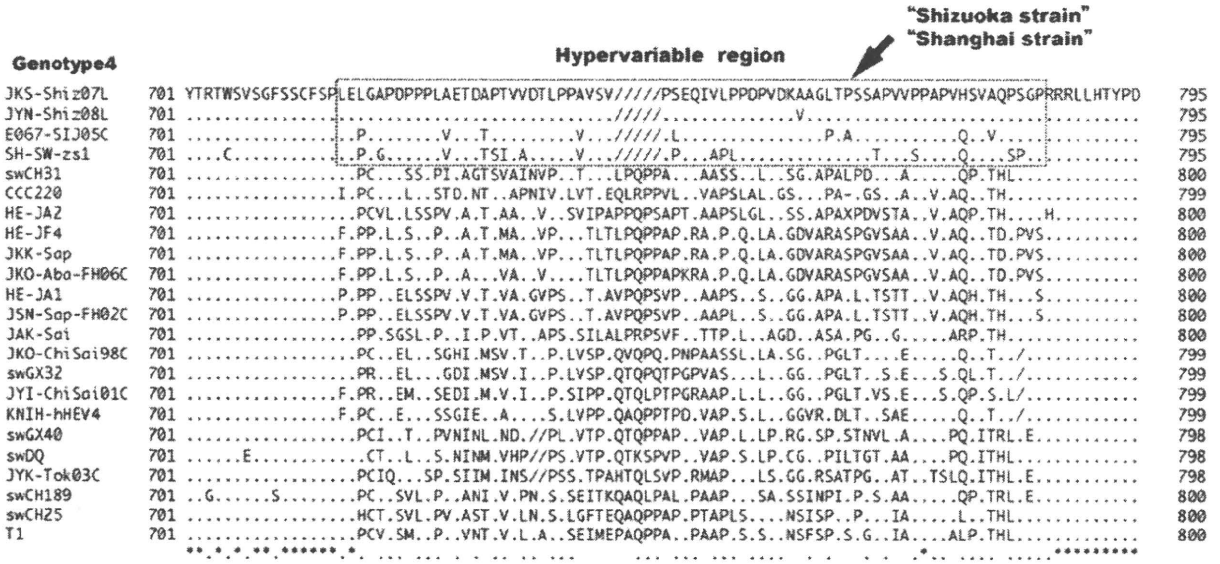

Fig. 2 Comparison of the amino acid sequences of hypervariable region in the ORF of hepatitis E virus genotype 4 isolates. The arrow indicates a box showing amino acid sequences of 'Shanghai strain' and 'Shizuoka strain'.

東海中部地区における genotype 4 HEV は中国から伝搬した可能性が存在する.

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Three cases of hepatitis E after eating deer meat or wild boar liver in West Shizuoka, Japan

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We experienced 3 cases of hepatitis E, where the patients developed their disease about two months after eating wild boar or deer captured in Shizuoka prefecture, Japan. All three were middle-aged men, and two ate raw wild boar's liver by chance in the same restaurant. One often ate the raw meat of deer in his son's hunting life. On admission, the hepatic dysfunction was mild in all of them (total bilirubin 1.0-10.0 mg/dl, AST 754-953 U/l, ALT 696-1182 U/l, prothrombin time 78-113%) and improved promptly with fluid infusion and rest. They were all positive for IgM and IgG HEV antibodies and HEV-RNA. Viral genome sequencing indicated that their HEV segregated to a cluster within genotype 4, with 99.8% or greater identity to each other. Interestingly, these isolates showed 98.5-99.8% identity to "Aichi Strain of HEV Genotype 4" previously recovered from humans and wild boars in Aichi prefecture, clearly different from those that are predominant in Japan, particularly in Hokkaido.

Key words: acute hepatitis　HEV　hepatitis E

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平成21年度　地域医療における疾病並びに医療等に関する研究調査(4)

兵庫県におけるHEV感染実態調査（第二報）

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	神戸大学医学部附属医学医療国際交流センター	矢野嘉彦（平成８年卒）
	神戸大学大学院微生物感染症学講座感染病理学分野	林祥剛（昭和56年卒）
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	東芝病院研究部	三代俊治

緒　言

Ｅ型肝炎ウイルス（HEV）が単なる輸入感染症の一種ではなく，国内固有株が存在することが見いだされてから早10年が経過した[1]．その間に，感染連鎖の中に動物が関与していること（人畜共通感染症）が判明し，徐々にその臨床像が明らかにされつつある[2]．我々も，2003年に鹿の生肉摂取によるＥ型肝炎集団発生例を経験し，人畜共通感染症であることを世界で初めて直接証明[3]して以降，その臨床像の解明に積極的に関与してきた[4-6]．

2006年に実施されたHEV感染に関する全国調査によって[2]，集積症例全体の約30％は動物由来感染，８％は輸入感染，２％は輸血を介する感染であることが判明したが，過半の症例（約60％）においては感染経路が不明のままであり，さらなる感染実態調査が必要とされている．

〈研究１〉

Ｅ型肝炎は不顕性感染や症状が軽微な症例も多いこと，抗体検査が未だに保険承認されていないこともあって，その感染実態は不明な点が多い．そこで，兵庫県におけるＥ型肝炎の感染状況，臨床像および感染ルートを明らかにする目的で，成因不明の急性肝炎症例を登録する兵庫県Ｅ型肝炎研究会を昨年度立ち上げ，今年度も継続調査を行った．

また，昨年度の調査では，従来の報告通りにＥ型肝炎症例が少なかったこともあり，集積された成因不明の急性肝炎症例全体の臨床像を明らかにすることも重要と考え，その最終診断・治療内容・転帰についても調査を行った．

〈研究２〉

2003年以降の野生猪HEV感染定点観測により，当地域の猪にHEVが高率に感染していること，その流行は少なくとも４年間遷延していること，猪・鹿・ヒトの間で共通のHEVが感染していることなどが判明した[5) 6)]．ところが2007年度以降に調査した猪は，血液中のHEV RNAがすべて陰性であった．当地域の野生猪におけるHEV感染は一時的なもので，このまま終息に向かう可能性が高いと推測している．

その一方で，静岡県ではこの数年間野生猪におけるHEV感染が問題として取り上げられ，猪肉摂取によるＥ型肝炎例が報告されている[7]．他の県でも，野生猪HEV感染の実態調査が既に実施あるいは実施予定と聞いている．従って，先行して定点観測を行ってきた当地域の感染が本当に終息するのか否かは，他の地域の調査に重要な示唆を与えるものと考えて，今年度も調査を継続した．

方　法

〈研究１〉

１）昨年度，兵庫県内診療諸施設に協力を求めて立ち上げた兵庫県Ｅ型肝炎研究会を今年度も継続し，成因不明の急性肝炎症例を登録して臨床的検討を加えた．血清2-5A合成酵素活性（2-5AS）は，SRLにて測定した．

２）東芝病院研究部に血清を集積して，血清HEV抗体（IgG，IgA，IgM）を測定し，HEV RNAの検出を試みた．

３）Ｅ型肝炎と診断された症例のみならず，それ以外の当初成因不明とされた急性肝炎症例全体で，最終診断，治療内容および転帰を登録各施設に照会

した.

〈研究２〉

兵庫県在住のハンターの協力を得て，兵庫県中部の地域において捕獲された野生の猪から肝臓と血液をサンプリングした．東芝病院研究部で血清 HEV-IgG 抗体を測定し，HEV RNA の検出を試みた．

結　果

〈研究１〉

１）2008年４月から2010年３月までの２年間に，兵庫県内17施設から69症例が登録された．その臨床像（表１）は，年齢中央値54歳，男女比は32：37とほぼ同数であった．肝障害の程度は軽度から高度まで様々な症例が集まったが，PT40％以下の重症例を５例含んでいた．2-5AS が基準値の100pmol/dl を越える症例は25例（37.3％）であった．

表１　成因不明急性肝炎69例の臨床像

年齢（中央値）	54	9-86
性別（男/女）	32/37	
AST (IU/l)	1,416	159-6,780
ALT (IU/l)	1,486	215-8,160
T-Bil (mg/dl)	4.9	0.4-35.0
PT (%)	77	7-116
2-5AS (pmol/dl)	177	29-4,570

２）今年度は HEV RNA 陽性例を１例認め，血清 HEV 抗体（IgG，IgA）も陽性であり，E 型急性肝炎と診断した．２年間で成因不明の急性肝炎症例69例中，E 型急性肝炎は２例（2.9％）と低率であった．IgG 抗体単独陽性例は３例（4.3％）であり，感染既往の可能性が高いと判断した．

今年度唯一，E 型急性肝炎と診断した症例は神戸市在住の46歳，男性．AST 5,580IU/l，ALT 8,920IU/l，T-Bil 3.1mg/dl，PT 39.8％．第９病日の採血で血清 HEV RNA 陽性，HEV 抗体（IgG，IgA）陽性，IgM 抗体陰性であった．HEV RNA は Genotype 4，中国株であった．PT が40％以下であったため，E 型急性肝炎（重症型）として慎重に経過観察されたが，保存的治療のみでそれ以上の悪化はなく，軽快退院した．詳細な問診により，２年程前から中国・上海駐在で，魚介類は普段からよく食べていること，発症２週間前（２月初め）に広州で鹿や猪の肉を火を通して食べた経緯が聴取された．

３）２年間で登録された成因不明の急性肝炎症例69例中，最終診断・治療内容・転帰が調査可能であったのは45例（65.2％）であった．

最終診断（疑診例を含む）は図１の通りで，最後まで不明のままであったのが19例（42.2％）と最も多かった．疑診例を含めて診断名で多かったのは，自己免疫性肝炎８例（17.8％），ウイルス性肝炎８例（17.8％），アルコール性肝障害５例（11.1％），薬剤性肝炎４例（8.9％）であった．ウイルス性肝炎として原因ウイルスが特定できたのは，C 型肝炎３例，E 型肝炎２例，A 型肝炎・EBV・HSV が１例ずつであった．

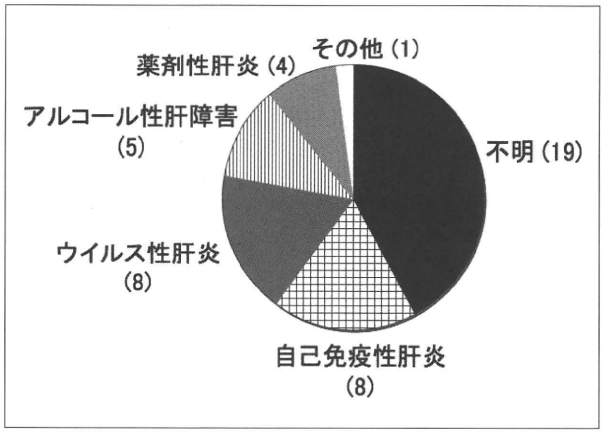

図１　成因不明急性肝炎45例の最終診断

治療内容は図２の通りで，保存的治療のみであったのが21例（46.7％）と最も多かった．積極的な治療方法で最も多かったのはステロイド治療11例（24.4％）で，ウルソやグリチルリチンで経過をみられた例も多かった．血液濾過透析や血漿交換は２例に行われた．

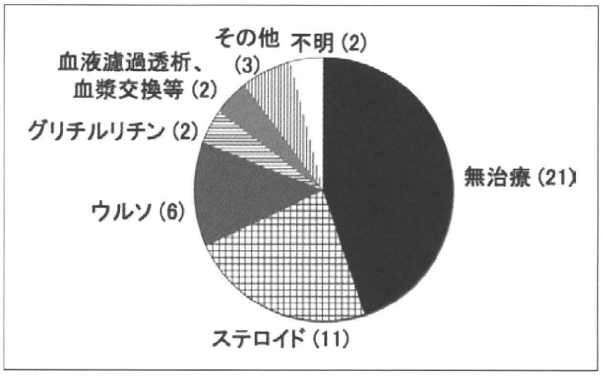

図２　成因不明急性肝炎45例の治療内容

転帰（図３）は，治癒が15例（33.3％），軽快25例（55.6％）と大部分は良好な経過を辿ったが，死亡例が３例（7.1％）にみられた．

〈研究２〉

今年度調査を行った猪19頭はすべて HEV RNA 陰

性であり，2007年度以降，HEV RNAが陽性あった猪は1頭も見られていない（表2）．

表2　野生猪におけるHEV感染実態調査

	n	HEV-RNA	Anti-HEV (IgG)
2003-2004年度	141	11 (7.8%)	33 (23.4%)
2005年度	39	0	13 (33.0%)
2006年度	102	5 (4.9%)	15 (14.7%)
2007年度	47	0	2(4.3%)
2008年度	88	0	7 (8.0%)
2009年度	19	0	ND
合計	436	16 (3.7%)	70 (16.8%)

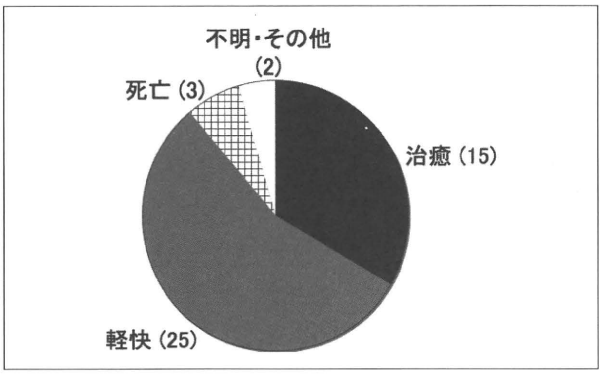

図3　成因不明急性肝炎45例の転帰

考　察

成因不明の急性肝炎の中でE型肝炎が占める割合は2.9%と極めて低率であった．先行している北海道E型肝炎研究会の報告[8]（13.5%）や国立病院機構共同研究班の全国調査[9]（1980年以降の調査で4.4%，2000年以降は10%内外）と比べても低く，これまで報告されている東高西低のHEV感染率を反映しているものと考える．

今年度唯一，E型急性肝炎と診断した症例も輸入感染例であり，それを除くと国内感染例と推定されるE型肝炎症例は昨年度の1例のみ（1.5%）と更に低率であった．来年度も調査を継続することで，兵庫県におけるE型肝炎の感染実態を明らかにしていく予定であるが，兵庫県における成因不明の急性肝炎症例の中でE型肝炎が占める割合は極めて低率であると推測された．

その一方で，当初成因不明とされた急性肝炎症例全体の臨床像を明らかにすることも重要と考え，最終診断・治療内容・転帰についても調査を行った．肝炎の原因が最後まで不明のままであったものが4割以上あり，診断がついたものでも疑診例が多かった．

自己免疫性肝炎は，特に急性肝炎様に発症した場合には典型的な血清学的所見や組織像を呈しない場合があり，診断に苦慮することが多い．また，ウイルス性肝炎のうちでもC型急性肝炎は，病初期には血清抗体が陰性あるいは低力価陽性であることが多く，診断に迷うことがある．さらに，アルコール性肝障害や薬剤性肝炎の診断には，他の原因の除外が必須であり，我々が以前に経験したE型肝炎症例も当初はアルコール性肝障害と誤診していた．

従って，急性肝炎の実地臨床において，例えその頻度が低くてもHEV感染の有無の確認を欠かすことは出来ない．E型肝炎の抗体検査が未だに保険承認されていない現状では，当調査を利用して積極的にHEVの関与を調べるように情報提供したい．

また，肝炎ウイルスはこれまでにHEVを含めて5種類が同定されているが，それ以外にも未知の肝炎ウイルスの存在する可能性が以前から指摘されている．今回の調査でも，RNAウイルス感染時に反応性に上昇することが知られている2-5ASを測定したところ，基準値の100pmol/dlを越える症例を約三分の一以上に認めたことは興味深い．肝炎の原因が最後まで不明のままであったもののうち，2-5AS高値の症例が7例あり，その中に未知の肝炎ウイルス症例が隠れている可能性があり，今後も積極的に症例の集積を続けていきたい．

治療内容に関しては，自然経過で軽快傾向を示す例が多かったこともあって，半数近くは無治療で経過をみられていた．ステロイド投与例が最も多かったのは，疑診例を含む自己免疫性肝炎例が多かったことと，原因不明の肝炎症例において免疫の関与を否定することが特に初期には困難なことなどが理由と考えられた．

転帰は，治癒あるいは軽快が9割近くと大部分は良好な経過を辿った．特別な治療的介入がなくても自然に軽快するウイルス性肝炎が含まれている可能性もあるが，HEVを含めた肝炎の原因の積極的な検索，自己免疫性肝炎例に対するステロイドの早期投与，重症例に対する血液濾過透析や血漿交換などが予後の改善に貢献しているものと考えた．

2007年度以降，HEV RNAが陽性あった猪は1頭も見られず，これまでのendemic areaでの積極的な狩猟がHEV感染の終息に貢献した可能性も推測される．猪におけるHEV感染が，少なくとも当地では，何らかの感染源から生じた一過性のものであった可能性が高く，静岡県を含めた他の地域での調査計画を立

てる上で，重要なヒントを与えるものと考える．

まとめ

昨年度立ち上げた兵庫県Ｅ型肝炎研究会を今年度も継続し，成因不明の急性肝炎症例の登録を継続した．２年間で17施設から69症例が登録され，このうち２例（2.9％）がＥ型急性肝炎であった．今年度唯一，Ｅ型急性肝炎と診断した症例も輸入感染例であり，兵庫県における成因不明の急性肝炎症例の中でＥ型肝炎が占める割合は極めて低率であると考えられた．

また，この７年間の野生猪HEV感染定点観測結果をみると，2007度以降，HEV RNAが陽性あった猪は１頭も見られず，当地での流行はこのまま終息する可能性が高いと考えられた．

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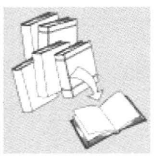
Reviews in Medical Virology

REVIEW



Efficient cell culture systems for hepatitis E virus strains in feces and circulating blood

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SUMMARY

Attempts have been made to propagate hepatitis E virus (HEV) in primary hepatocyte culture and various other cultured cells. However, the replication ability of HEV recovered from culture media remains extremely low. Recently, efficient culture systems have been established in PLC/PRF/5 (hepatocellular carcinoma) and A549 (lung cancer) cell lines for HEV strains of genotypes 3 and 4 in our laboratory. They originated in fecal extracts from patients containing HEV RNA in extremely high-titers (10^7 copies/ml), and named the JE03-1760F (genotype 3) and HE-JF5/15F (genotype 4) strains, respectively. HEV RNA in culture supernatants reached 10^8 copies/ml in titer, and were transmitted successively through many passages. An infectious HEV cDNA clone (pJE03-1760F/wt) was constructed that has replication activity comparable to that of the wild-type JE03-1760F in feces. The ORF3 protein is indispensable for shedding HEV particles from cells in the reverse genetics system. HEV recovered from culture media, as well as circulating HEV, possess ORF3 proteins on the surface and are covered with cellular membranes, and therefore, ORF2 epitopes are buried in these particles. In contrast, HEV excreted into feces are naked nucleocapsids without a lipid layer or surface expression of the ORF3 protein. HEV in sera of patients with acute hepatitis E can infect and replicate in PLC/PRF/5 and A549 cells, with efficiency comparable to the circulating HEV RNA levels. High-efficiency cell culture systems for infectious viruses, thus developed, are expected to open up a new era and resolve many mysteries in the epidemiology, molecular biology, and treatment of HEV. Copyright © 2011 John Wiley & Sons, Ltd.

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INTRODUCTION

The hepatitis E virus (HEV) can induce a spectrum of liver disease in human beings ranging from acute through severe-acute to fulminant hepatitis at the extreme end. HEV is a non-enveloped small virus with a diameter of 27–34 nm (mean: 30 nm), and is classified into the genus *hepevirus* of the family *Hepeviridae* [1]. The genome of HEV is a single-stranded, positive-sense RNA composed of 7200 nt, and has a cap structure at the 5′-end as well as a poly(A) tail at the 3′-end, and possesses short untranslated regions at 5′- and 3′-termini and three open reading frames named ORF1, ORF2, and ORF3 [2], respectively (Figure 1). ORF1 codes for non-structural proteins such as methyltransferase, papain-like cysteine protease, helicase, and RNA-dependent RNA polymerase, while ORF2 encodes the viral capsid protein. ORF3 codes for a small, phosphorylated protein made of 113 or 114 aa [3–10]. ORF1 protein is translated from the genomic RNA, while ORF2 and ORF3 proteins from a bicistronic subgenomic RNA of 2.2 kb in length [11,12]. HEV principally replicate in the liver, are shed into the intestinal lumina via the bile duct, and are subsequently excreted into feces. Therefore, HEV can spread easily and widely in circumstances with low sanitary conditions, by a fecal–oral route, and it is highly prevalent in developing countries in Asia, Africa, and Central America that are located in the tropical and subtropical zones.

Until very recently, HEV was regarded to cause a rare "imported infection" in developed countries,

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Abbreviations:
aa, amino acids; DOCA, deoxycholic acid; HAV, hepatitis A virus; HEV, hepatitis E virus; mAb, monoclonal antibody; nt, nucleotide(s); MOI, multiplicity of infection; RT-PCR, reverse transcription-polymerase chain reaction

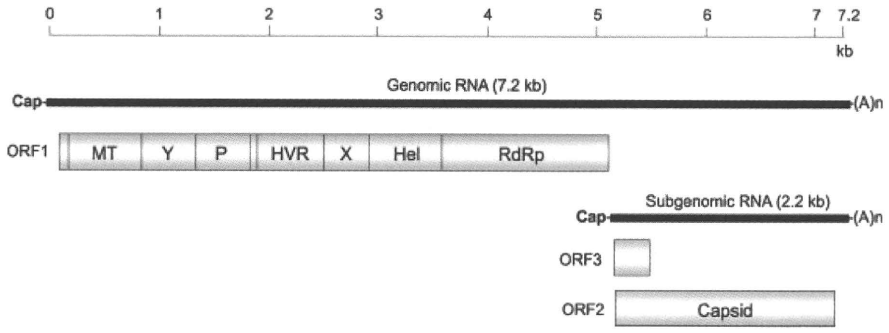

Figure 1. The genomic structure of HEV. MT, methyltransferase; Y, Y domain; P, papain-like protease; HVR, hypervariable region; X, X domain; Hel, helicase; and RdRp, RNA-dependent RNA polymerase

including the United States, European countries, and Japan, and has consequently not attracted much attention. Toward the end of the 1990s, however, HEV started to be recognized in patients with acute hepatitis in the United States and Europe, who had not traveled to endemic areas within a reasonable incubation period of up to 6 weeks [13–17]. In 1997, the first animal strain of HEV, swine HEV, was identified and characterized in pigs from the United States [18]. Beginning in 2001 in Japan, sporadic acute hepatitis E cases have started to be reported increasingly more often [19–21]. Such incidents instigated the survey of pigs in farms throughout Japan, and wide-spread HEV infections were identified in the animals, which became public knowledge [22–24]. Furthermore, outbreaks of HEV infection in individuals who had consumed meat and/or internal organs of pigs or wild animals (boars and deer) in the recent past were also reported [25–29]. Public attention to HEV culminated when fatal cases of severe-acute or fulminant hepatitis E were reported [30–33]. Thus, hepatitis E suddenly was a topic of interest, and this interest promoted rapid advances in research on the diagnosis and epidemiology of HEV infections. Japan was among the first to note the importance of zoonosis in the maintenance and spread of HEV among the community [23,25,26,30,34].

Although HEV has only one serotype, it is classified into four genotypes designated from 1 to 4; they are distinguished by nt differences of >25% in the entire genomic sequence [34–36]. Genotypes 1 and 2 infect humans exclusively, and are associated with infectious hepatitis in endemic areas, while genotypes 3 and 4 infect not only humans, but also animals such as domestic pigs and wild boars. They are responsible for sporadic acute hepatitis as a result of zoonotic HEV infection (Table 1) [34,35,37]. Very recently, a novel strain of HEV was isolated from rabbits farmed in China [38]. The rabbit HEV is most closely related to HEV genotype 3, with approximately 82% nucleotide sequence identity, suggesting that rabbit HEV may be a variant of genotype 3. However, there is little information regarding the host range and zoonotic potential of the rabbit HEV. At least three genotypes of avian HEV have been identified in chickens around the world, but they share merely ~50% of the nucleotide sequence with mammalian HEV genotypes [37,39].

Virological studies on HEV have been hampered by the lack of *in vitro* culture systems that support of its replication. Our laboratory succeeded in establishing efficient cell culture systems for infectious HEV, which has opened up new research for analyzing the virus itself that has not been possible since the discovery of the virus by Balayan *et al.* [40]. This article reviews the road map toward the establishment of infectious HEV in cell cultures, and introduces new knowledge that has evolved by the virtue of this system.

PROPAGATION OF INFECTIOUS HEV IN CULTURED CELL LINES

Five hepatitis viruses are known, and they are named using capital letters from A to E [41]. Of the five types, cell culture systems have been established for HAV by Provost *et al.* [42], and applied to the production of an inactivated hepatitis A vaccine over the world. For HCV, an *in vitro* culture system was worked out by Wakita *et al.* [43] with use of an infectious cDNA clone of a JFH-1 strain of genotype 2a originating in serum

Table 1. Epidemiology and clinical characteristics of the four genotypes of HEV

Genotype	Distribution	Transmission	Countries		Clinical characteristics
			Developing	Developed	
1	Asia Africa	Water-borne (epidemic)	Frequent	Rare (imported)	Prevalent in younger people
2	Mexico Africa[a]				Aggravation in pregnant women
3	Worldwide (except in Africa)	Food-borne (sporadic)	Rare	Frequent	Zoonotic infections (in pigs, wild boars, and deer)
4	China, India Indonesia Japan, Taiwan Vietnam				Prevalent in middle-aged and elderly men Severe-acute and fulminant hepatitis in middle-aged and elderly men Chronic infections in recipients of organ transplantation

[a]Egypt, Chad, Namibia, the Central African Republic and Democratic Republic of the Congo are included.

from a patient with fulminant hepatitis C. It invited robust advances in research on the infectious capacity, replication, and particle formation of HCV. No cell culture systems have thus far been established for hepatitis B virus or hepatitis delta virus strains from clinical samples: virus particles released into the culture supernatant are not infectious and cannot be passaged through cell cultures [44–47]. Attempts to cultivate HEV have been made by many researchers since the 1980s [48–57]. However, the replication efficiency of HEV has remained extremely low, making it difficult to passage the viruses through multiple generations, which prohibited analyses of their physicochemical structures and replication mechanisms. However, the replication of HEV had been observed in cell lines transfected with RNA transcripts of infectious cDNA clones, as well as a replicon derived from the virus [58,59]. Monkeys inoculated with culture media or lysates of cells transfected with HEV-replicon developed infections, but their viral titers were low.

The breakthrough occurred using fecal sample from a sporadic case of acute hepatitis in Japan containing a very high titer of HEV RNA (JE03-1760F strain of genotype 3: 2.0×10^7 copies/ml) that opened up a new road toward establishing the culture system for HEV. Using fecal extract as an inoculum, the replication capacity of HEV was evaluated in 21 established cell lines derived from humans, monkeys, cows, dogs, rats, and mice, including three human hepatocellular carcinoma cell lines (HepG2, Huh7, and PLC/PRF/5 cells). As the results, the JE03-1760F strain was discovered to replicate efficiently in two cell lines; PLC/PRF/5 (Alexander) from hepatocellular carcinoma and A542 originating in lung cancer [60]. Daughter viruses were excreted into culture media in titers reflective of the HEV inoculum. Thus, when 8.6×10^5 copies [multiplicity of infection (MOI): approximately 0.9] were seeded per well in the six-well plate, HEV in the culture medium reached 10^8 copies/ml on day 60 (Figure 2). HEV

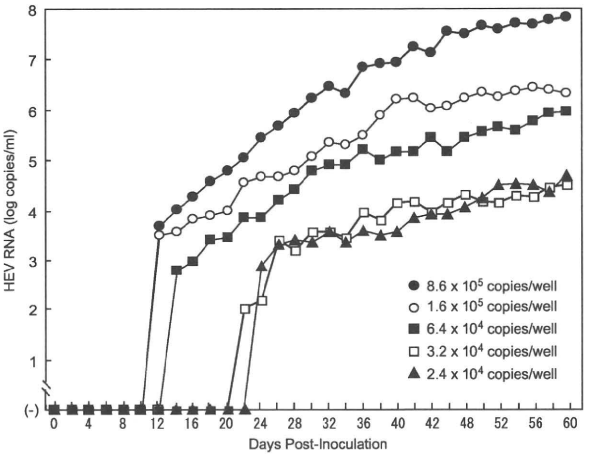

Figure 2. Replication of HEV in the PLC/PRF/5 (Alexander) cell line originating in hepatocellular carcinoma. HEV RNA titers in culture media are plotted for wells seeded with decreasing copy numbers of fecal HEV (JE03-1760F strain of genotype 3) until 60 days of culture. Copied from Reference [60] with permission

was detected in media even when the inoculum size was reduced to 2.4×10^4 (MOI: 0.03) per well. Moreover, daughter HEV in media replicated efficiently through many passages in PLC/PRF/5 and A542 cell lines. The first successful cultivation of the virus may have been due to the extremely high HEV titer in the inoculum and 29 point mutations (6 non-synonymous mutations that caused aa changes) possessed by the wild-type virus in feces, which were different from all previously reported genotype 3 HEV strains [61].

The initial success was followed by establishment of the culture system with use of another inoculum. It was a fecal extract from a patient with fulminant hepatitis E containing the HE-JF5/15F strain of genotype 4 in a high titer (1.3×10^7 copies/ml) [62]. Just as was observed with the JE03-1760F strain, the HE-JF5/15F strain could be propagated successively, and a daughter strain (HE-JF5/15F_p5) was detected at a concentration of 10^4 copies/ml in culture media on the 5th passage at day 2, and the titer reached 1.5×10^6 copies/ml on day 10 (Figure 3). HEV of genotype 4 is associated with aggravation of hepatitis on the basis of epidemiological data [31,63]. A high replication activity of HEV genotype 4, reproduced in the culture system for the HE-JF5/15F strain of this genotype, is expected to shed light on a role of viral factors in the development of fulminant hepatitis E in some patients who have been infected with virus.

ADAPTATION AND POINT MUTATIONS OF HEV DURING CELL CULTURE

For the purpose of characterizing the genetic mutations involved in the adaptation of HEV to the environment of cell cultures, passage experiments in two arms (A and B) were conducted using the JE03-1760F strain as the initial inoculum [64]. In experiment A, cultures were passaged 10 times, until the inoculum size was10^5 copies/well. The median time elapsed from the inoculation to the appearance of HEV in culture media was shortened by 1 week for 6–10 passages in comparison with 0–5 passages (10.0 days vs. 16.7 days); a HEV RNA titer of 10^5 copies/ml was achieved by a median of 19 days earlier for 6–10 than 0–5 passages (16.0 days vs. 35.2 days). HEV strains with a higher replication activity were selected successively in passages 11, 12, and 13 with a decreasing inoculum size (30, 10 and 3×10^2 copies/well, respectively). As a result, the HEV strain (p13/A), recovered after the highest number of passages, was able to infect at a virus

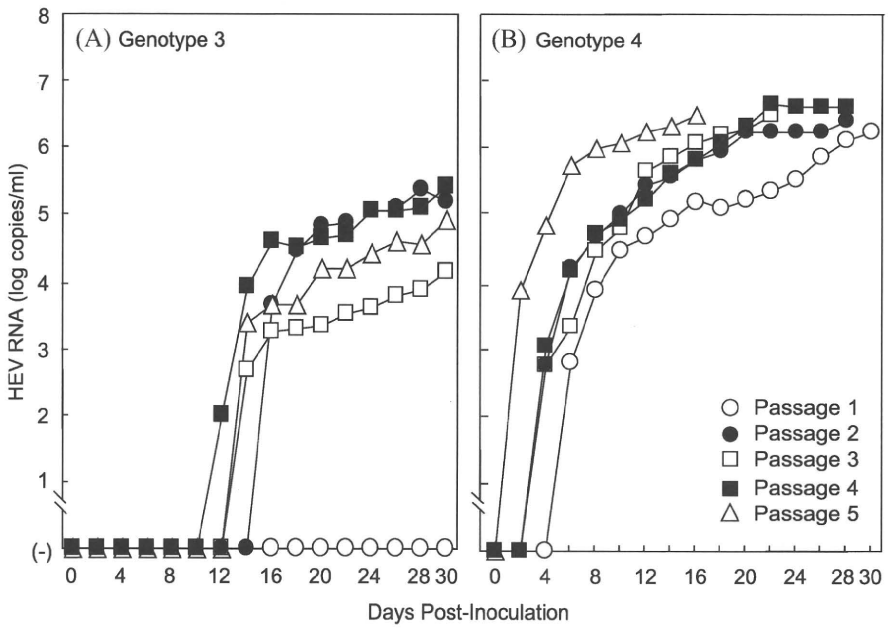

Figure 3. Propagation of HEV genotype 3 (JE03-1760F) and HEV genotype 4 (HE-JF5/15F) in the PLC/PRF/5 cell line in culture. Excretion patterns of (A) genotype 3 HEV derived from the feces of a patient with acute hepatitis E, and (B) HEV genotype 4 from that of a patient with fulminant hepatitis E are compared through 5 passages until 30 days of culture

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load 1/30 that of the parent wild-type strain (JE03-1760F in feces). In experiment B, a shorter duration before the appearance of HEV in media was also achieved after 6–10 compared to 0–5 passages. Eventually, HEV became detectable after only 2 days following the seeding in culture media for 5–10 passages, with the time before virus titers had reached 10^5 copies/ml shortened by the mean of 1 week (17.0 days vs. 9.6 days), and the concentration of HEV in media exceeded 10^6 copies/ml 10 days after inoculation of the 10th passage [64].

Full-genome sequences of a 13-passage strain (p13/A) in experiment A and a 10-passage strain (p10/B) in experiment B were determined and compared with that of the parent wild-type HEV (Table 2). The newly derived strain had mutations in 19 and 23, respectively, of the 7226 nt in the genome. Of the 19 variations found in p13/A, 5 were non-synonymous mutations in ORF1 (3 of them) and ORF3 (2). Likewise, of the 23 variations identified in p10/B, 10 were non-synonymous mutations in ORF1 (4 of the 10), ORF2 (3), and ORF3 (3).

REVERSE GENETICS SYSTEMS FOR ENGENEERING INFECTIOUS HEV

Using the genomic RNA obtained from a fecal specimen containing the JE03-1760F strain, as the template, a full-length infectious cDNA clone (pJE03-1760F/wt) was constructed [65]. Three cDNA fragments covering the entire genome, one of which contained the T7 RNA polymerase promoter sequence upstream of the extreme 5′-end in the JE03-1760F genome, were amplified by RT-PCR, and inserted into the *Hin*dIII and *Bam*HI sites of a plasmid vector, along with Fragment poly-AT7Φ carrying 31 nt of adenine and a T7 terminator sequence (Figure 4A). Then, using the *Nhe*I-linearized plasmid as a template, the genomic RNA was transcribed *in vitro*, a cap was added at the 5′-end, and the RNA was transfected into PLC/PRF/5 cells in culture. HEV RNA in culture media started to increase on day 4, and was maintained at high levels ($>10^7$ copies/ml) from days 28 to 60 (Figure 4B). A cDNA clone of the wild-type virus deprived of ORF1 (pJE03-1760F/ΔORF1), whish served as a negative control, did not replicate in PLC/PRF/5, and HEV RNA levels kept decreasing during the culture period.

The ORF2 protein in PLC/PRF/5 cells was stained by immunofluorescence with a mouse anti-ORF2 mAb (H6225) [66]. Cultured cells staining positive for ORF2 protein increased with time over days 5, 7, 11, and 15, reflecting a spread in HEV infection in these cells (Figure 4C). HEV in the supernatant of cultured fostering cells transfected with RNA transcripts of pJE03-1760F/wt, not only infected PLC/PRF/5 as well as A549 cells in culture, but also could be passaged through many generations retaining a replication capacity comparable to that of the wild-type JE03-1760F strain in patient's feces.

AN ORF3-NULL MUTANT CLONE FOR ANALYSIS OF THE FUNCTION OF THE ORF3 PROTEIN

The function of the ORF3 protein of HEV remains unknown. Various functions have been attributed to ORF3 in cells in which it is over-expressed [67]. For example, the ORF3 protein has been suggested to interact with cellular proteins, including signal proteins containing Src homology 3 domains [68], bikunin [69], hemopexin [70], and microtubule proteins [71], and it might modulate the acute-phase disease response [72], protect cells from mitochondrial depolarization [7] and enhance the expression of glycolytic-pathway enzymes [6]. However, its function under the physiological conditions is not clear. Nor it is known whether or not the ORF3 protein is required for the morphogenesis and release of virus particles. Recent studies using infectious HEV cDNA clones have suggested that the expression of intact ORF3 protein is essential for infection of animals [73,74]; however, it is not required for infection and virion morphogenesis *in vitro* [75]. For the purpose of exploring the function of the ORF3 protein, HEV cDNA clones with and without ORF3 expression were compared for their impact on virion egress in cell culture. A cDNA clone harboring a defect in ORF3 (pJE03-1760F/ΔORF3) was produced by converting its initiation codon (AUG) to GCA. A549 cells inoculated with the ORF3-defective virus did not shed HEV particles into culture media (Figure 5A), although they produced intracellular HEV RNA at levels comparable to those of the parent wild-type clone (pJE03-1760F/wt; Figure 5B). On the basis of these observations, the ORF3-null mutant was confirmed to replicate in cells, but nonetheless, is not able to direct the infected cells to secrete HEV extracellularly.

Table 2. Comparison of the nucleotide sequences of wild-type JE03-1760F and two daughter strains (p13/A and p10/B) over the entire genome

Nt no.	Region	Nt			aa	
		Wild-type	p13/A	p10/B	Residue no.	Mutation
61	ORF1	U	U	C[a]	12	—
370	ORF1(MT)[b]	C	U	C	115	—
445	ORF1(MT)	U	U	C	140	—
591	ORF1(MT)	C	U	C	189	Ala to Val
829	ORF1(Y)	C	C	U	268	—
1378	ORF1(P)	C	C	U	451	—
1549	ORF1(P)	U	U	C	508	—
2191	ORF1(HVR)	C	C	U	722	—
2236	ORF1(HVR)	C	C	U	737	—
2246	ORF1(HVR)	U	C[c]	C	741	Trp to Arg
2704	ORF1(X)	U	C	U	893	—
2808	ORF1(X)	U	U	C	928	Val to Ala
2913	ORF1(Hel)	A	A	G	963	Glu to Gly
2915	ORF1(Hel)	G	G	U	964	Val to Leu
2938	ORF1(Hel)	C	U	C	971	—
3106	ORF1(Hel)	A	G	A	1027	—
3223	ORF1(Hel)	U	U	C	1066	—
3235	ORF1(Hel)	C	U	C	1070	—
3453	ORF1(Hel)	C	U	C	1143	Ala to Val
3475	ORF1(Hel)	C	C	U		
3496	ORF1(Hel)	C	U	C	1157	—
3553	ORF1(Hel)	C	U	C	1176	—
3620	ORF1(Hel)	U	C	U	1199	—
4015	ORF1(RdRp)	C	U	C	1330	—
4309	ORF1(RdRp)	C	C	U	1428	—
4462	ORF1(RdRp)	C	U	C	1479	—
5312	ORF2	U	U	C	47	—
	ORF3				51	Ile to Thr
5378	ORF2	A	G	G	69	—
	ORF3				73	Asn to Ser
5456	ORF2	C	U	U	95	—
	ORF3				99	Pro to Leu
6047	ORF2	U	U	C	292	—

(*Continues*)

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Table 2. (Continued)

Nt no.	Region	Nt			aa	
		Wild-type	p13/A	p10/B	Residue no.	Mutation
6470	ORF2	C	U	C	433	—
6578	ORF2	C	U	C	469	—
6611	ORF2	C	U	C	480	—
6626	ORF2	U	C	U	485	—
6651	ORF2	G	G	R[d]	494	Val to Ala/Thr
6652	ORF2	U	U	C	494	Val to Ala/Thr
6855	ORF2	A	A	G	562	Asn to Asp
6944	ORF2	U	U	C	591	—
7186	3′ UTR	C	C	U	NA	

[a] Nt mutations are shaded for visual clarity.
[b] MT, methyltransferase; Y, Y domain; P, papain-like protease; HVR, hypervariable region; X, X domain; Hel, helicase; RdRp, RNA-dependent RNA polymerase.
[c] Nt mutations that are commonly seen in the two distinct series of passages in experiments A and B, are shown in bold type.
[d] R, mixture of A and G.

The expression of the ORF3 protein was examined by immuno-capture PCR with a mouse anti-ORF3 mAb (TA0536) [76]. ORF3 protein was not detectable on HEV particles in the patient's feces. In contrast, the protein was identified on HEV particles excreted into the media of cultured cells [76,77]. In the absence of detergent, only a small percentage of the HEV particles were captured by anti-ORF2 and anti-ORF3 antibodies. This stands in a sharp contrast to the fact that both anti-ORF2 and anti-ORF3 antibodies were able to capture virtually all of the HEV particles after they had been treated with various detergents. These lines of evidence point to the existence of ORF3 protein on HEV particles produced in culture, but that the protein is covered with a lipid layer.

HEV particles in the feces are not captured by a mAb specific to ORF3 protein. They are released from the liver through the bile duct into the intestinal lumina. During this transition, HEV particles are exposed to deoxycholic acid (DOCA) in the bile and proteolytic enzymes excreted from the pancreas. It is reasonable to presume, therefore, that fecal HEV are deprived of ORF3 protein and free of cellular membranes constituting the envelope. In support of this view, HEV particles in culture media, after treatment with DOCA and trypsin, were trapped completely by the mAb to ORF2 protein, but not at all by that to ORF3 protein. In line with these observations, the density of cultured HEV in sucrose gradient centrifugation shifted from 1.15–1.16 to 1.27–1.28 g/cm^3 (Figure 6D), after treatment with DOCA and trypsin, which was comparable to that of fecal HEV (Figure 6A). Of note, the buoyant density of HEV particles treated with 1% DOCA (Figure 6C), or 5% Tween 20, or even after they had been treated with 10% chloroform, shifted to 1.20–1.25 g/cm^3, which was still a little lower than 1.27–1.28 g/cm^3, suggesting that proteolytic digestion is necessary to dissociate ORF3 protein from HEV particles released from infected cells.

The results of these experiments have extensively characterized the ORF3 protein. The protein is a structural HEV protein, in that it exists on the surface of nascent HEV. As such, it most likely has a critical role in the release of HEV from infected cells. HEV particles in culture media are covered with cellular membranes and behave as if they were "enveloped" viruses. In contrast, HEV particles excreted into feces are naked "non-enveloped" virus as has been documented in text

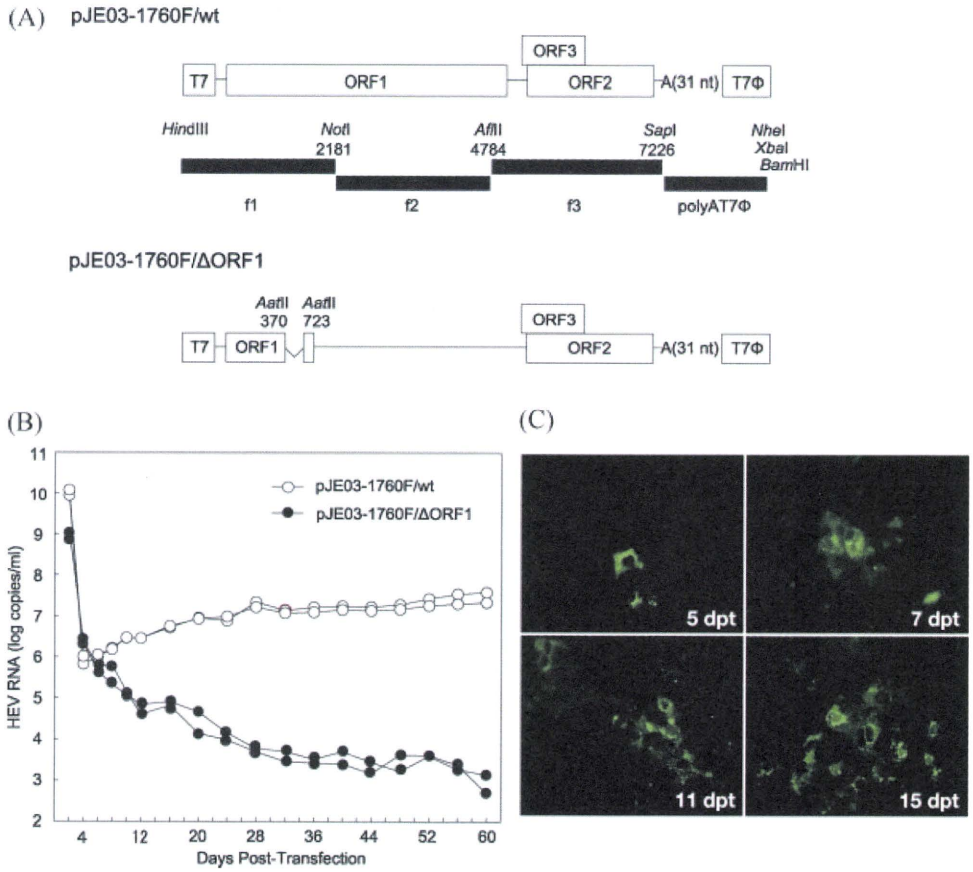

Figure 4. Production and replication of an infectious HEV cDNA clone. (A) Construction of the wild-type cDNA clone (pJE03-1760F/wt) and the same clone deprived of ORF1 (pJE03-1760F/ΔORF1). (B) Replication of these two clones, representing the wild-type HEV and a negative control, is compared in the culture meida of the PLC/PRF/5 cell line transfected with these clones. (C) Expression of the ORF2 protein in cultured cells transfected with the wild-type HEV cDNA clone was followed until 15 days post-transfection by staining with the mAb against the ORF2 protein labeled with Alexa Fluor 488. Copied from Reference [65] with permission

books for several decades [78]. In fact, HEV exists in two different forms, one "enveloped" and the other "non-enveloped." Hence, the presence of "enveloped" HEV in the physiological state was sought for further experiments.

INFECTIOUS CAPACITY OF HEV STRAINS IN CULTURES DERIVED FROM PATIENT SERA

HEV particles from patient sera, in the absence or presence of anti-HEV, have a density peaking at 1.15–1.16 g/cm^3 in sucrose gradient centrifugation, similar to those in culture media (Figure 6E,F). HEV particles in culture media, unlike those in feces, take the form of an "enveloped" virus. Nonetheless, they can infect cultured cells, and can replicate efficiently. On the other hand, cases of post-transfusion HEV infection have been reported from Japan [79–81] and other countries [82–84]. These lines of evidence invite a possibility for establishing an HEV culture system with use of circulating HEV particles as the starting inoculum.

Sera from 23 patients with hepatitis E, who had been exposed to either imported or domestic HEV infections, were seeded into PLC/PRF/5 cells in culture [85]. Each well of the six-well plate received HEV of genotype 1, 3, or 4 either in the order of 10^6 copies [1.5–3.0 $\times$ 10^6 copies/well ($n = 4$)] or that of 10^5 copies [1.5–5.8 $\times$ 10^5 copies/well ($n = 9$)]. HEV replicated in all the wells, to levels reflecting the size of the inoculation. Thus, HEV RNA titers increased to the median of 2.1 $\times$ 10^6 copies/ml for wells seeded with HEV in the 10^6 order, and to 1.9 $\times$ 10^5 copies/ml for wells seeded with HEV in the 10^5 order on day 30 (Figure 7A,B, respectively). HEV replicated in 8 of the 19 (42%) wells receiving

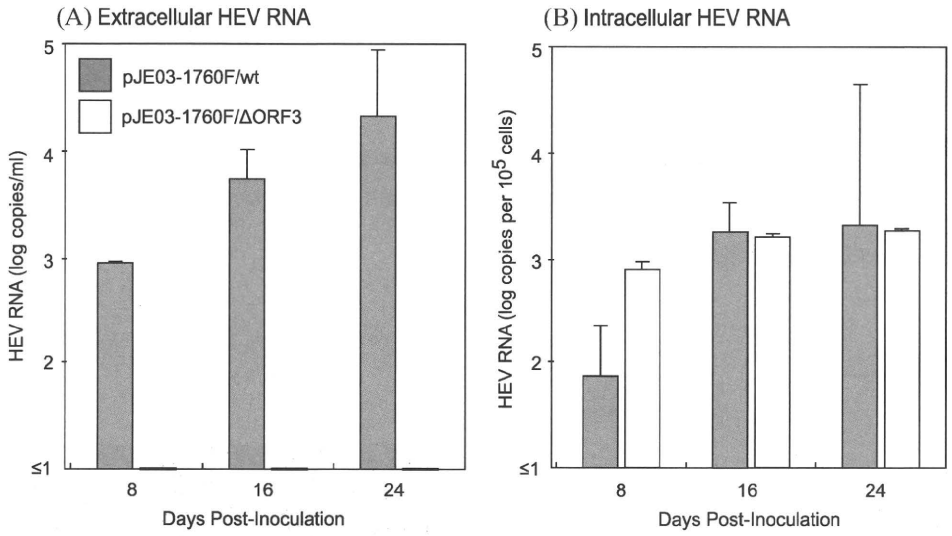

Figure 5. Production of HEV by cells in culture inoculated with viruses derived from HEV cDNA. Patterns of the wild-type cDNA clone (pJE03-1760F/wt) and the same clone deprived of ORF3 (pJE03-1760F/ΔORF3) are compared. (A) Extracellular secretion of HEV into culture media. (B) Intracellular production of HEV. See Reference [77] for further details

HEV in the order of 10^4 ($2.0–7.2 \times 10^4$ copies/well), and HEV RNA levels in these wells reached a median of 3.9×10^4 copies/well (Figure 7C). Of interest, genotype 3 HEV strains tended to replicate more efficiently than genotype 4 HEV strains, with a higher median HEV load on day 30 (1.7×10^6 copies/ml vs. 6.7×10^4 copies/ml on day 30) in the culture supernatant of wells receiving HEV in the 10^5 order (Figure 7B) along with a higher frequency of infection (6/12 or 50% vs. 2/7 or 29%) in wells receiving HEV in the 10^4 order (Figure 7C). These genotype-dependent behaviors stand at a substantial variance with our previous experiences on fecal HEV; the genotype 4 strain (HE-JF5/15F) replicated much more efficiently than the genotype 3 strain (JE03-1760F) (Figure 3) [62]. It remains to be seen, therefore, which of genotypes and strain differences affects the efficiency of replication to higher extents.

The nucleotide sequence spanning 412 nt in ORF2 was determined, and the sequence was compared between the serum HEV used as the inoculum and the HEV in culture media recovered at day 30. The 100% identity between these ORF2 sequences testified to the successful propagation of circulating HEV in culture. When HEV in culture media were seeded into A549 cells, they replicated efficiently and raised HEV RNA levels to 1.0×10^7 copies/ml in culture media [85].

Of the 21 serum samples for which the replication of HEV was confirmed, only three were negative for anti-HEV (marked by asterisks in Figure 7). HEV in the remaining 18 (86%) serum samples co-existed with anti-HEV. Irrespective of co-occurring anti-HEV, the density of HEV in the samples peaked at 1.15–1.16 g/cm^3, that is, the same as that of HEV in culture media (Figure 6E,F). When immuno-precipitation with goat anti-human IgG/IgM/IgA antibodies was performed on HEV from 4 serum samples with and 3 samples without anti-HEV, the HEV stayed mostly in the supernatant and only 8.1% of the samples were recovered in precipitates. In contrast, fecal HEV samples, which had been pre-incubated with sera from patients in the recovery phase of acute hepatitis E, were precipitated nearly completely by anti-human immunoglobulin antibodies. Based on these results, serum HEV does not form immune complexes with anti-HEV and circulate in a free form [85].

Pre-treatment of HEV in sera with detergent, such as Tween 20 and NP-40, made them reactive with anti-ORF2 and anti-ORF3 mAb to some extent, when enabled them to be recovered in precipitates, as was observed for HEV in culture media. When HEV in sera were pre-treated with detergents and proteolytic enzymes, they banded at 1.27–1.28 g/cm^3 in a sucrose gradient, to the

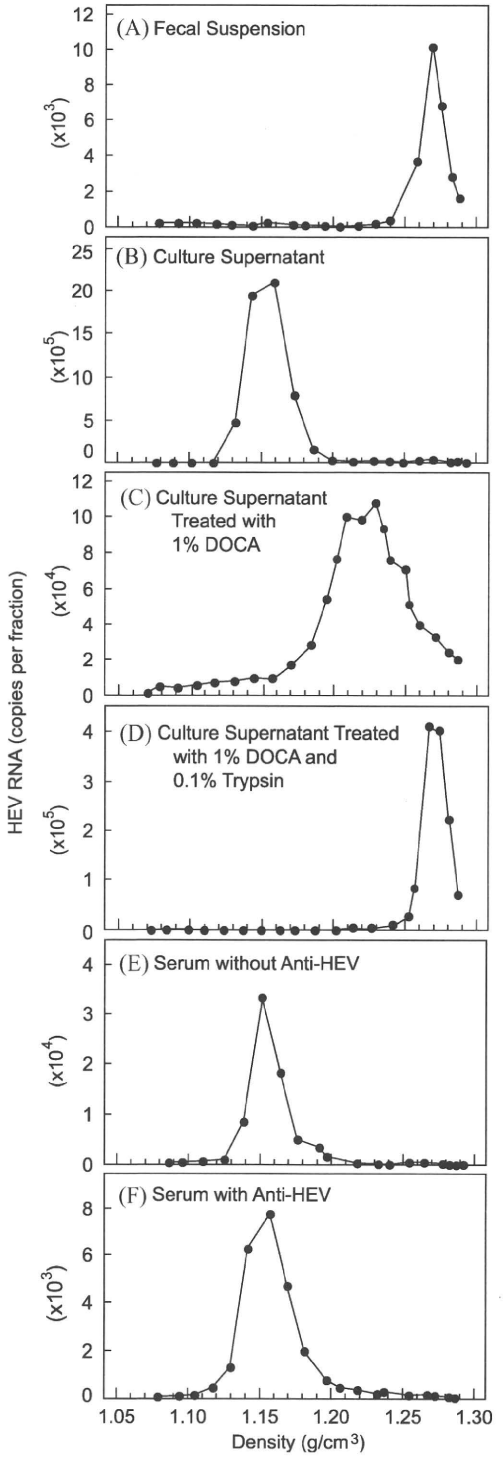

Figure 6. The density of HEV in sucrose gradient centrifugation. HEV from various sources are compared (A–F). The effects of treatment with detergent, either alone or in combination with proteases, are evaluated for HEV recovered from culture media (C, D)

same density as the fecal HEV. At the same time, they became completely capturable by anti-ORF2, but could not be captured by anti-ORF3 at all. Thus, by treatment with detergents and proteolytic enzymes, circulating HEV can gain the same properties as HEV in feces, just as was observed for HEV generated in cell cultures. Therefore, HEV in culture media, in titers much higher than circulating HEV, were evaluated for their infectious capacity after they had been treated with detergents and proteolytic enzymes. HEV in culture media did not lose infectious activity after they had been treated with detergents in the presence or absence of proteolytic enzymes [85].

All through the above studies, HEV inocula with higher HEV RNA titers infected culture cells the more efficiently. This holds true irrespective of the source of inoculum, including fecal HEV (JE03-1760F of genotype 3 as well as HE-JF5/15F of genotype 4) and circulating HEV with or without co-occurring anti-HEV. Circulating HEV can infect culture cells efficiently, provided that approximate HEV RNA titers in serum exceed 5.0×10^5 copies/ml. As mentioned above, HEV excreted into culture media are covered with cellular membranes and have a density lower than that of fecal HEV. Likewise, circulating HEV are also covered with cellular membranes, and are endowed with the structure of an "enveloped" virus. As a result, co-existing anti-HEV antibodies do not influence the infectious activity of circulating HEV that have been propagated in culture. At the present moment, however, it has not been explained how "non-enveloped" viruses like fecal HEV and "enveloped" viruses as the HEV in culture media and sera infect cultured cells. The crystal structure of HEV-like particles was confirmed recently, with their outer surface (a target for neutralizing antibodies) mainly constructed by the middle and protruding domains [86–89]. However, a putative receptor for HEV has not been identified, and virtually nothing is known about the mechanism by which HEV enters susceptible cells. It is conceivable that "non-enveloped" HEV infects target cells through specific interaction between viral capsid and host receptor molecule. For "enveloped" HEV, there may be another as-yet-unknown mechanism for entry to hepatocytes, since neither ORF2 nor ORF3 protein is exposed on its surface. The mechanism of absorption and entry both of these "non-enveloped" and "enveloped"

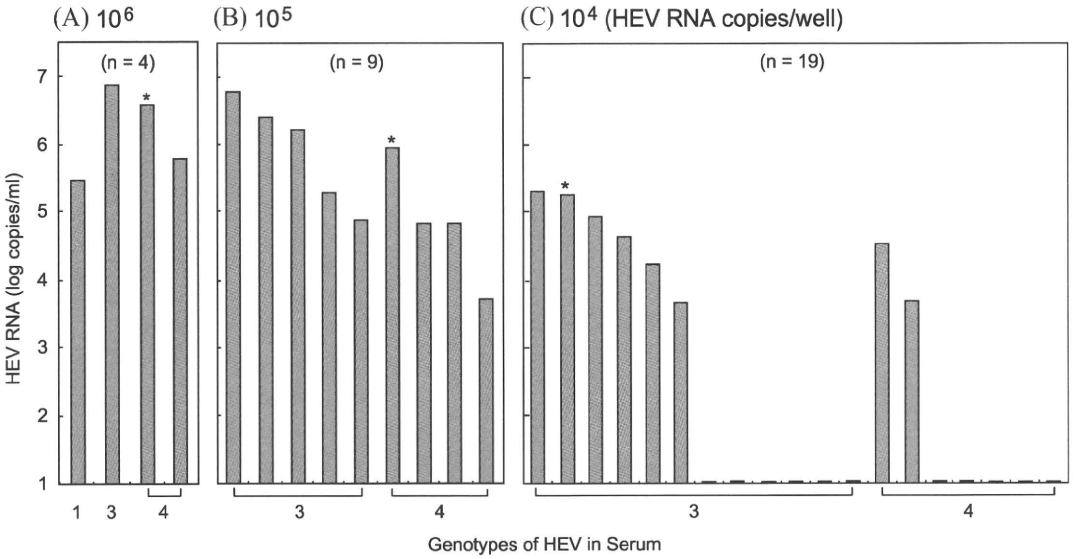

Figure 7. Secretion of HEV by PLC/PRF/5 cells infected with HEV from sera. HEV RNA titers in the supernatant of wells inoculated with (A) 10^6 copies/well, (B) 10^5 copies/well, and (C) 10^4 copies/well were compared on day 30. The frequencies of HEV secretion were 100% (4/4) for 10^6 wells, 100% (9/9) for 10^5 wells, and 42% (8/19) for 10^4 wells. Genotypes of HEV strains propagated in culture are specified as 1, 3, or 4 at the bottom. Asterisks represent the sera from patients seronegative for anti-HEV

HEV into hepatocytes, as the first step of infection, as well as virus shedding from hepatocytes into circulation, need to be clarified. Such studies are required in order to better understand the life cycle of HEV.

CONCLUSION

For the first time, cell culture systems were developed capable of secreting infectious HEV in high titers into culture media. The success with the original JE03-1760F strain has been extended to other strain that can bring about the replication of HEV with an even higher efficiency, and that can be passaged through many generations. Further, infectious HEV cDNA clones have been engineered. These cell culture systems, reinforced by reverse genetics, will solve many mysteries and answer numerous questions surrounding the epidemiology, viral absorption/entry, packaging and delivery of viral particles, toward illuminating the life cycle of HEV.

Efficient propagation of authentic HEV in feces and circulation, as well as HEV cDNA clones, in PLC/PRF/5 and A549 cells is expected to encourage the application of this system for other viruses for which a culture has not yet been established. A key to successful propagation in culture would be a high infectious capacity of primary inocula, that is, the concentration of virus in copies/ml.

The discovery of "enveloped" HEV is not unprecedented for infectious hepatitis viruses that have been believed to represent the naked nucleocapsid. It has been reported that some HAV particles excreted into culture medium are associated with lipids and are non-neutralizable [90,91]; however, their association with lipids is not to the extent of HEV in cell culture and blood circulation, essentially all of which are now found to be "enveloped." New knowledge about HEV has invited novel questions as well. For example, it is not known why HEV in culture media and sera can infect hepatocytes, even though they are "enveloped," with a virulence comparable to that of "non-enveloped" HEV in feces. It is also unclear whether anti-HEV can protect hepatocytes from infection with HEV introduced by transfusion, even though the viruses are "enveloped" and not reactive with antibodies. These mysteries need to be addressed and resolved in future studies in which absorption and entry of "enveloped" HEV will be defined more precisely. The solution is within reach, now that an efficient *in vitro* infection system for HEV is in our hands.

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