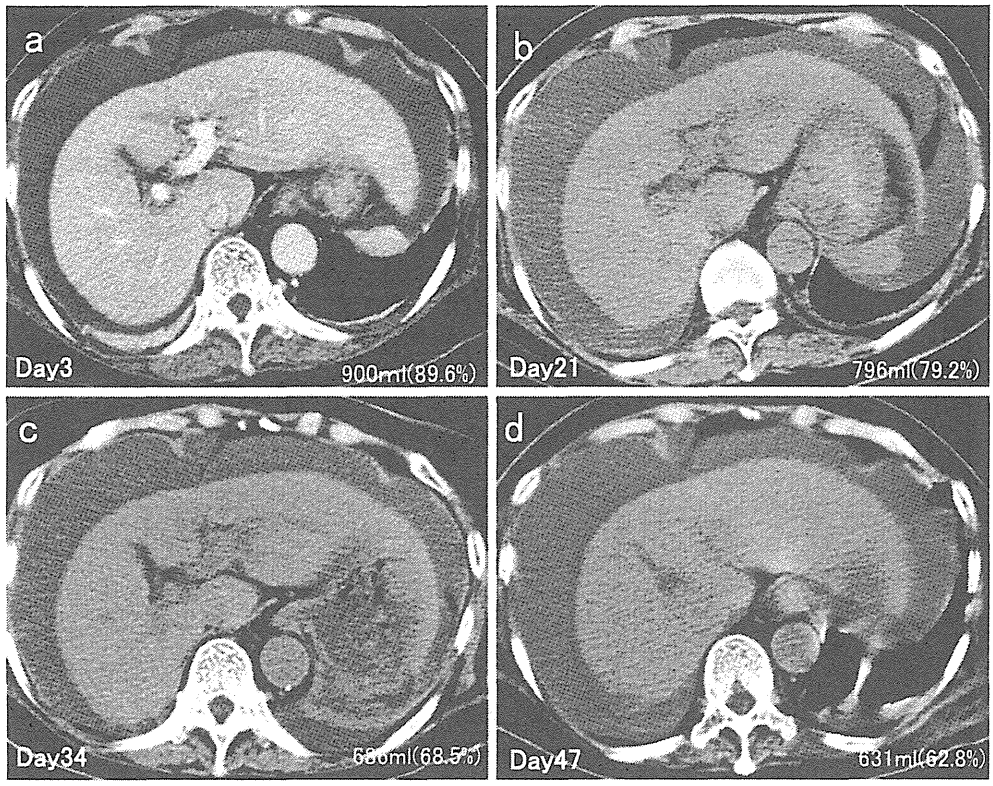

Fig. 2　Abdominal CT
Enhanced CT on day 3 (a) showed liver atrophy and appearance of ascites. Plain CT on day 21 (b), day 34 (c), day 47 (d) showed progression of liver atrophy. Calculated standard liver volume using the method of previous report was 1004 m*l*. Liver volume estimated with CT was 900 m*l* (89.6%) on day 3, 796 m*l* (79.2%) on day 21, 688 m*l* (68.5%) on day 34, and 631 m*l* (62.8%) on day 47.

病理組織学所見で肝には広範な肝細胞壊死を認め小葉構築は不明瞭化し，残存する肝組織は僅少であった．肝細胞は変性，出血，壊死を認め，門脈域には軽度から中等度のリンパ球浸潤も認めた（Fig. 4）．

HEV 感染指標の検討，遺伝子系統解析

2例とも HAV, HBV, HCV 急性感染所見を認めないため，北海道E型肝炎研究会に症例登録を行い，病初期（症例1は第3病日，症例2は第1病日）血清を用いて HEV 感染診断を行った．HEV 感染は，HEV genome open reading frame（ORF）2領域における RT-PCR 法による HEV RNA 同定[11)12)]，enzyme linked immune sorbent assay（ELISA）キット（特殊免疫研究所，東京）による抗 HEV IgM，IgAの検出により診断した．HEV 遺伝子型は，HEV ORF 1 の metyltransferase 領域内 326 塩基に対する sequencing により決定し，遺伝子系統解析は unweighted pair-grouping method with arithmetic means（UPGMA 法）により行った．ELISA による抗 HEV IgA 抗体，IgM 抗体，IgG 抗体は2症例のいずれにおいても陽性で，HEV RNA 陽性，Genotype4 であった．本2症例から得られた HEV 2株（JFI-Hak10, JFS-Hak10）は，HEV ORF1 326 塩基に対する遺伝子系統樹解析で，09年札幌小流行の成因となった“new Sapporo strain”に属し，互いに隣接していた（Fig. 5）．さらにこれら2例の連続血清を用いて既報の如く血中 HEV RNA 定量を行った[5)]．症例1では血中 HEV RNA は入院時 5.6 log copies/m*l* であったが，第19病日には 3.1 log copies/m*l* 迄低下していた．症例2では血中 HEV RNA は入院時 5.8 log copies/m*l* であったが，その後減衰せず，観察可能であった第50病日まで 6 log copies/m*l* 前後で変動し推移した（Fig. 6）．

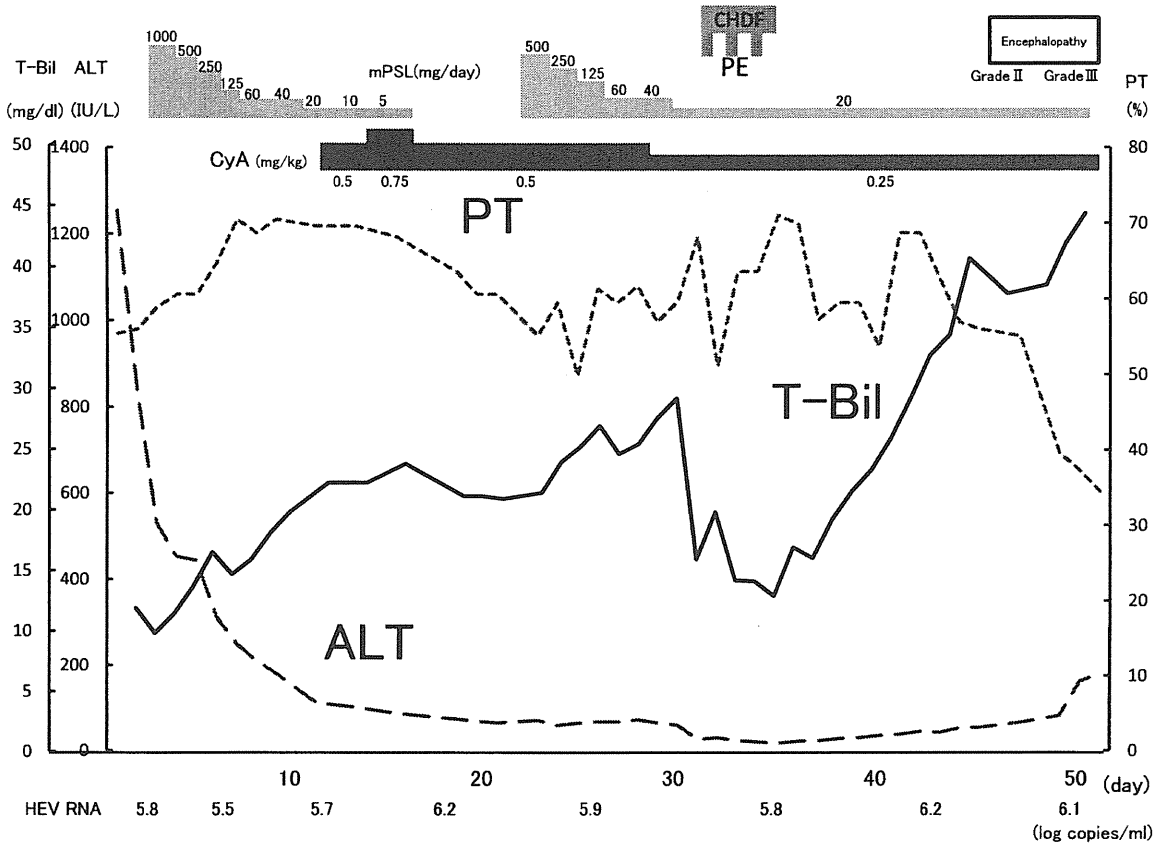

Fig. 3　Clinical course of the patient 2
(mPSL: methylprednisolone, CyA: cyclosporine A, PE: plasma exchange, CHDF: continuous hemodiafiltration)

考　察

2009年秋, genotype 4 HEV 単一系統株"new Sapporo strain"によるE型急性肝炎の札幌小流行が観察された[7][8]. 札幌圏でほぼ同時的に発症し診断された患者11名のうち1名は重症型急性肝炎を示したものの劇症化例を認めなかった. HEV 感染経路の探索については5名は豚内臓肉摂取歴を有したが, 7名では認めず全例に共通する感染経路は不明と報告された. さらに2009年12月に茨城県から北海道（千歳, 浦河, 苫小牧地区）への旅行歴がある男性が2010年1月に急性E型肝炎を発症し自然寛解したが, 分離されたHEV RNA は遺伝子型4に属し遺伝子系統解析では "new Sapporo strain" に含まれると報告された[9].

著者らは2010年春に, HEV 遺伝子型4の"new Sapporo strain"に属する同一株の感染により, ほぼ同時期に発症し劇症化した函館在住の2症例を経験した. 2名は, 何れも函館地区に居住し, HEV 感染推定時期に札幌を含む道央圏への旅行歴はない. 2症例ともに明らかな豚内臓肉摂取歴を認めず, 互いに交流し或は食事に同席する機会を持たなかったことから感染源は不明であった. しかし, 遺伝子系統解析により分離 HEV 株（JFI-Hak10, JFS-Hak10）に著しい近縁性が示されたこと（Fig. 5）および発症時期の同一性から, E型肝炎2例は何れも函館地区に流通する HEV 汚染食品を摂取して HEV に感染した可能性が考えられた. HEV "new Sapporo strain" に函館地区で感染した本2例の背景には, HEV で汚染した生鮮食品が道央圏から供給され函館地区にも流通した可能性の他に, 函館とその近郊に既に "new Sapporo strain" が循環する感染源が存在する可能性も排除できない. 札幌から300 km 以上遠隔の函館地区における "new Sapporo strain" 伝播が生じたのであれば, 今後札幌のみならず北海道内他地域における同株起因E型肝炎が発症する可能性も示唆された. 今後も遺伝子系統解析手法を用いた分子疫学的検討により HEV 感染経路の探索が求められると考えられた.

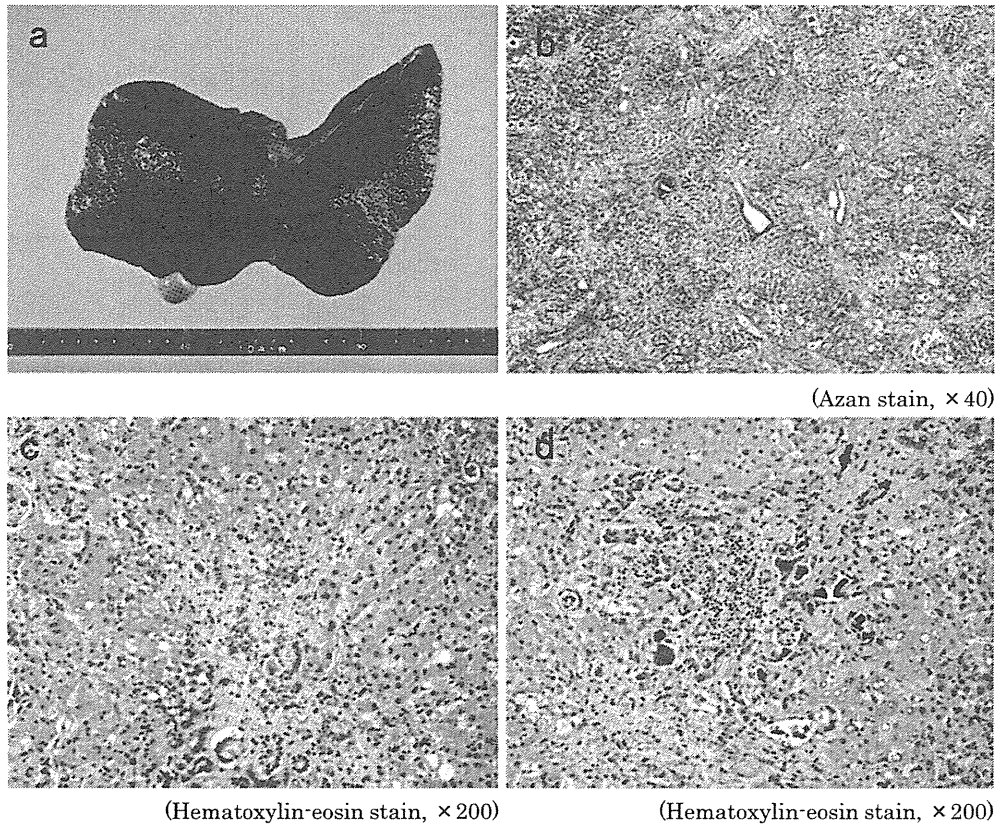

Fig. 4 Pathological findings of the autopsied liver
Liver atrophy was advancing, with a liver weight of 625 g, in the pathological anatomy (a). Massive hepatocellular necrosis was observed and the lobules had significantly shronk in liver (b). Degeneration, bleeding, and necrosis were observed in the centrilobular region of the liver (c). Increased cholangiole and cholestasis were observed at the peripheral portal area, and lymphocytic infiltration was also observed.

E型急性肝炎の重症化に関連するウイルス側因子として，genotype 4感染[13]があげられ，宿主側因子としては妊娠[14]，高齢[13]，背景肝疾患[15]，糖尿病などの基礎疾患[1]が指摘されている．HEV “new Sapporo strain”は前述の如くgenotype 4に属するが著者らの2例は本HEV株感染例では初めての劇症例である．E型急性肝炎全国集計254例に基づく阿部らの報告[12]によれば，自然寛解したE型急性肝炎の平均年齢は52.8歳だが，劇症肝炎では58.9歳と有意に高齢であった．本2例も63，73歳と高齢であり，さらに死亡した症例2は症例1に比して10歳年齢が高く糖尿病も合併した．本例で高齢，糖尿病合併が劇症化に関連したか否か，関連したとすればどのようなメカニズムが介在したかは不明であるが，高齢者では肝炎発症後の肝再生過程が遷延ないし不良となる可能性は存在するものと推測された．症例2では高度の糖尿病からNASHを背景肝疾患として有していた可能性も考えられるが，通院歴もなく病理組織学的にも組織構築の破壊が著明で背景肝疾患の推定は困難であった．

一般的にE型急性肝炎において血中HEV RNAは経過中早期に減少しHEV感染は一過性と考えられている[16]．しかし2008年臓器移植後のレシピエントにおいてHEV感染の慢性化が報告された[17]．この報告はフランスの移植施設における後ろ向きコホート調査で217人の移植患者の14人（6.5%）にHEV感染がおこり，そのうち8人（57.1%）が慢性化していた，という報告である．その後も臓器移植後や免疫抑制剤投与下での慢性化の報告が散見され[18][19]，さらには急速に肝硬変に進展する症例も報告された[20]．これらはいずれも免疫抑制状態という特殊な生体環境下の報告であり今回の劇

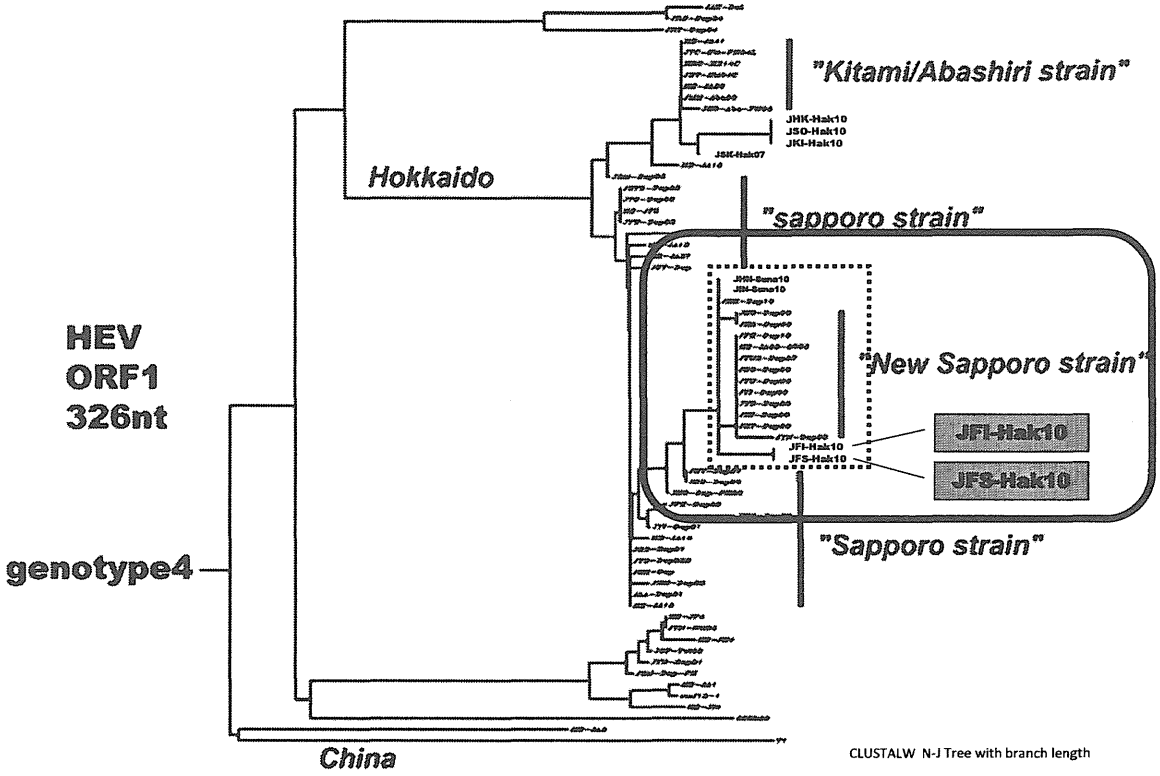

Fig. 5 Phylogenetic analysis for HEV strains isolated from the current 2 cases of fulminant hepatitis E
Phylogenetic tree for the partial nucleotide sequences of the open reading frame (ORF) 1 region (326nt) of HEV is shown. JFI-Hak10 and JFS-Hak10 separated from the cases presented were adjacent to each other, belonging to the "new Sapporo strain" of genotype 4 HEV responsible for the small epidemic occurred in Sapporo, Hokkaido 2009.

症肝炎の病態とは異なるが，HEV RNA が排除されない病態で肝炎が持続する可能性が示された事は本2例の臨床経過を考察する上で非常に興味深い．症例1では経過中に血中 HEV RNA は順調に減少し良好な経過を辿った（Fig. 1）が，症例2においては経過中に HEV の減衰を認めず，肝炎沈静化を期待して施行したステロイドパルス療法等によっても肝炎はなお遷延し発症53日目に肝性脳症を合併し肝不全で死亡した(Fig. 3)．症例2におけるウイルス血症の遷延化に免疫抑制療法が関与したかは不明である．現在までに E 型肝炎に対してステロイドホルモンを投与し血中 HEV 量を観察しえた症例は Matubayashi[5] らによる輸血後 E 型肝炎の1例のみと思われる．そこでは血中 HEV 量は predonisolone 投与による影響は受けていない．また，ステロイドホルモンやシクロスポリン療法により E 型急性肝炎慢性化が実証された報告はない．症例2では入院時 HbA1c 11.9% と未治療であった糖尿病が免疫応答を低下させ，ウイルス排除が障害された結果ウイルス血症が持続し，肝炎の遷延化に関与した可能性が考えられた．前述した E 型慢性肝炎の治療法としては，インターフェロン療法[21)22)]やリバビリン療法[23)]の既報があり，E 型急性肝炎に対してはインターフェロン療法の報告が散見される[5)24)]が，最近重症 E 型急性肝炎に対してリバビリン療法が有効であったとの1例報告もある[25)]．著者らが経験した症例2では抗ウイルス療法は施行していない．病態が既に進行した後に初期血清に対する HEV 感染検討を行ったため HEV 感染診断が遅れたこと，HEV RNA 定量は保存した連続血清に対する retrospective な解析で，本症例に特異的と思われる HEV 血症持続は後日知り得た事実である為である．real time な HEV 感染診断とその後の血中 HEV モニタリングを背景に抗 HEV 療法が施行されたなら，症例2では転帰が異なった可能性が存在する．今後重症化した E 型急性肝炎における抗ウイルス療法の適応，方法についても検討が

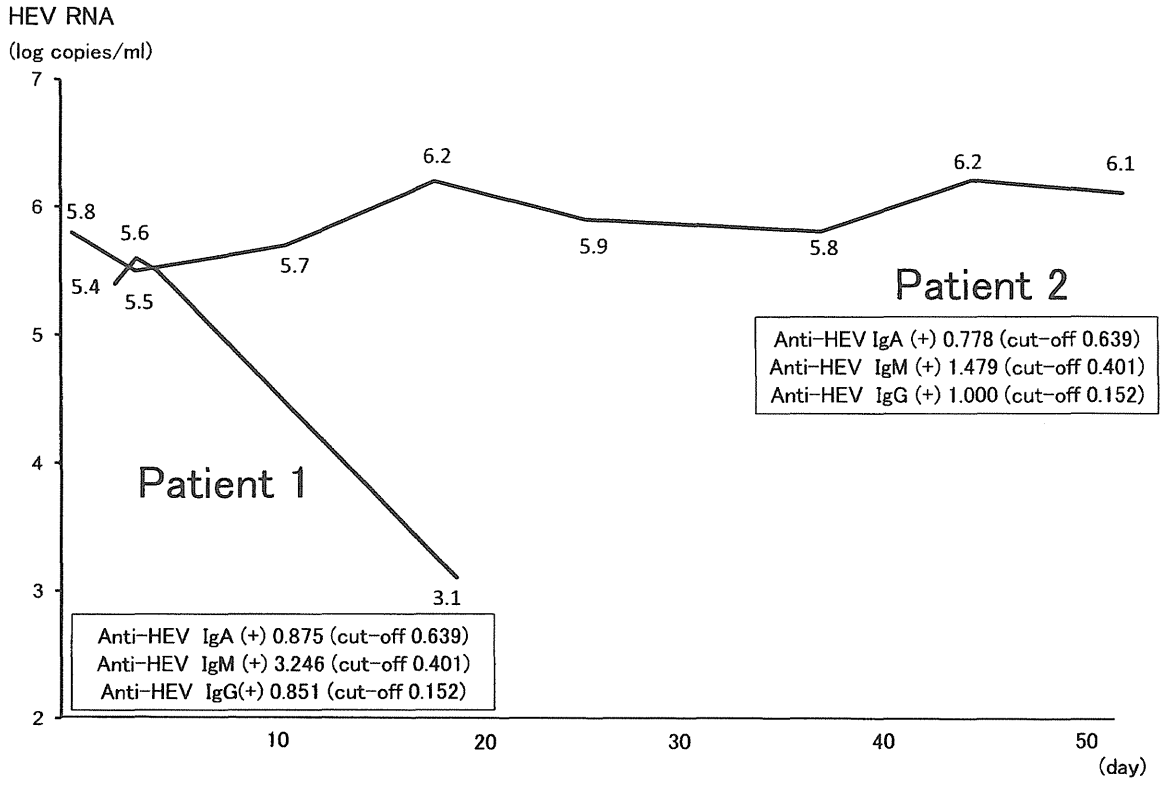

Fig. 6 Changes of levels in HEV RNA quantified in sera in 2 patients
A rapid decrease of quantified HEV RNA following admission to the hospital was observed in sera obtained from the Patient 1, but viremia over 5 log copies/m*l* had continued during 50 days in Patient 2. Anti HEV IgA, IgM and IgG exhibited positive in the sera obtained on Day 3 in patient 1 and on Day 1 in patient 2, respectively.

必要であるが，その為には簡便なHEV感染診断が重要である．2011年10月から保険診療の範囲でも使用可能となった抗HEV IgA測定系の速やかな普及が望まれるところである．さらに，重症型E型急性肝炎では速やかなHEV genotype診断が求められ，劇症化が危惧される症例では血中HEV RNA定量によるHEV動態の解析をもとに抗HEV治療の導入を要すると考えられた．

文　献

1) Mizuo H, Yazaki Y, Sugawara K, et al. Possible risk factors for the transmission of hepatitis E virus and for the severe form of hepatitis E acquired locally in Hokkaido, Japan. J Med Virol 2005; 76: 341—349
2) Ohnishi S, Kang JH, Maekubo H, et al. Comparison of clinical features of acute hepatitis caused by hepatitis E virus (HEV) genotypes 3 and 4 in Sapporo, Japan. Hepatol Res 2006; 36: 301—307
3) Ohnishi S, Kang JH, Maekubo H, et al. A case report: two patients with fulminant hepatitis E in Hokkaido, Japan. Hepatol Res 2003; 25: 213—218
4) 加藤　将，種市幸二，松林圭二．焼肉店での会食後に発生したE型肝炎ウイルス集団感染　うち1例は劇症肝炎で死亡．肝臓　2004；45：688
5) Matsubayashi K, Kang JH, Sakata H, et al. A case of transfusion-transmitted hepatitis E aused by blood from a donor infected with hepatitis E virus via zoonotic food-borne route. Transfusion 2008; 48: 1368—1375
6) 菅原伸明，矢和田敦，高橋和明，他．劇症E型肝炎：“Kitami/Abashiri strain”が検出された第3例目．肝臓　2009；50：473—474
7) 姜　貞憲，松居剛志，佐賀啓良，他．Genotype4単一系統株による札幌圏内E型肝炎小流行．肝臓　2010；51：51—53

8) 小関　至，姜　貞憲，水尾仁志，他．2009年秋に札幌圏で発生したE型肝炎小流行の臨床的・ウイルス学的・分子疫学的解析．肝臓　2012；53：78—89
9) 相川達也，池澤和人，間宮　孝，他．札幌圏内小流行4型HEV株が検出された茨城県内E型肝炎の1例．肝臓　2010；51：579—581
10) Urata K, Kawasaki S, Matsunami H, et al. Calculation of child and adult standard liver volume for liver transplantation. Hepatology 1995; 21: 1317—1321
11) Takahashi K, Iwata K, Watanabe N, et al. Full-genome nucleotide sequence of a hepatitis E virus strain that may be indigenous to Japan. Virology 2001; 287: 9—12
12) Takahashi K, Kang JH, Ohnishi S, et al. Full-length sequences of six hepatitis E virus isolates of genotypes III and IV from patients with sporadic acute or fulminant hepatitis in Japan. Intervirology 2003; 46: 308—318
13) 阿部敏紀，相川達也，赤羽賢浩，他．本邦に於けるE型肝炎ウイルス感染の統計学的・疫学的・ウイルス学的特徴：全国集計254例に基づく解析．肝臓　2006；47：384—391
14) Patra S, Kumar A, Trivedi SS, et al. Maternal and fetal outcomes in pregnant women with acute hepatitis E virus infection. Ann Intern Med 2007; 147: 28—33
15) Hamid SS, Atiq M, Shehzad F, et al. Hepatitis E virus superinfection in patients with chronic liver disease. Hepatology 2002; 36: 474—478
16) Emerson SU, Purcell RH. Hepatitis E virus. Rev Med Virol 2003; 13: 145—154
17) Kamar N, Selves J, Mansuy JM, et al. Hepatitis E virus and chronic hepatitis in organ-transplant recipients. N Engl J Med 2008; 358: 811—817
18) Kamar N, Mansuy JM, Cointault O, et al. Hepatitis E virus-related cirrhosis in kidney-and kidney-pancreas-transplant recipients. Am J Transplant 2008; 8: 1744—1748
19) Haagsma EB, van den Berg AP, Porte RJ, et al. Chronic hepatitis E virus infection in liver transplant recipients. Liver Transpl 2008; 14: 547—553
20) Gerolami R, Moal V, Colson P, et al. Chronic hepatitis E with cirrhosis in a kidney-transplant recipient. N Engl J Med 2008; 358: 859—860
21) Kamar N, Abravanel F, Garrouste C, et al. Three-month pegylated interferon-alpha-2a therapy for chronic hepatitis E virus infection in a haemodialysis patient. Nephrol Dial Transplant 2010; 25: 2792—2795
22) Haagsma EB, Riezebos-Brilman A, van den Berg AP, et al. Treatment of chronic hepatitis E in liver transplant recipients with pegylated interferon alpha-2b. Liver Transpl 2010; 16: 474—477
23) Kamar N, Rostaing L, Abravanel F, et al. Ribavirin therapy inhibits viral replication on patients with chronic hepatitis E virus infection. Gastroenterology 2010; 139: 1612—1618
24) 江林明志，井上和明，高橋和明，他．インドから帰国直後に重症E型肝炎を発症した日本人妊婦の1例．肝臓　2009；50：60—64
25) Gerolami R, Borentan P, Raissouni F, et al. Treatment of severe acute hepatitis E by ribavirin. J Clin Virol 2011; 52: 60—62

Two cases of fulminant hepatitis E occurred in Hakodate area 2010, suggesting infection with single source hepatitis E virus separated from small epidemic in Sapporo 2009

Koji Ogawa[1]*, Yoshiya Yamamoto[1], Machiko Umemura[2], Jong-Hon Kang[3], Hidekatsu Sakata[4], Keiji Matsubayashi[4], Kazuaki Takahashi[5], Masahiro Arai[5], Shunji Mishiro[5]

In April 2010, two elder female patients presented fulminant hepatitis E in Hakodate city, Hokkaido, Japan. HEV strains identified in their blood sampled belonged to “new Sapporo strain” of Genotype 4 separated from the small epidemic happened in Sapporo 2009, showing significant similarity to each other by phylogenetic analysis. One patient of 63-year-old improved rapidly, however, another one of 73-year-old died from prolonged course of hepatitis. The quantified levels of HEV RNA in sera had promptly declined in the former, but those had sustained over 5 log copies/m*l* during 7 weeks in the latter. Lasting viremia in HEV infection might be associated with prolonged liver injury in the latter patient.

Key words: hepatitis E　hepatitis E virus　fulminant hepatitis　New Sapporo Strain

Kanzo 2012; 53: 206—215

1) Department of Gastroenterology, Hakodate Municipal Hospital
2) Department of Internal Medicine, Hakodate Central General Hospital
3) Center of Gastroenterology, Teine Keijinkai Hospital
4) Hokkaido Red Cross Blood Center
5) Department of Medical Sciences, Toshiba General Hospital
*Corresponding author: k-ogawa@hospital.hakodate.hokkaido.jp

＜短　報＞

既報日本株よりは中国長春株に近い塩基配列のgenotype 4 HEVが検出された山口県内イノシシ肉生食後E型急性肝炎の1例

沖田　幸祐[1)*]　高橋　和明[2)]　原田　克則[1)]　谷岡ゆかり[1)]　平野　厚宜[1)]
木村　輝昭[1)]　加藤　　彰[1)]　山下　智省[1)]　新井　雅裕[2)]　沖田　　極[1)]

緒言：E型肝炎ウィルス（HEV）日本固有株の存在が報告された2001年以降，国内感染例が相次いで発見され，2006年には本邦におけるHEV感染の全国集計が初めて報告された[1)]．Zoonotic food-borne transmissionの重要性が認識され，急性肝炎診断の際に，多発地域への海外渡航歴がなくとも加熱不充分の動物肉あるいは内臓の摂取歴があればE型肝炎が鑑別診断として考慮されるようになった．

今回，山口県西部地区において自宅で調理した野生イノシシ肉あるいは肝が感染源として疑われた1例を経験し，患者血清から分離したHEVが興味深い塩基配列を有していたので報告する．

症例：39歳男性．生来健康．自営業を営んでおり，アルコールはビール350 ml/日程度で喫煙歴はない．身長171 cm，体重66 kg．2011年2月中旬に山口県下関市豊田地区で狩猟されたイノシシ肉を譲り受け，家族とともに生食した．その中にはレバーも含まれていた．

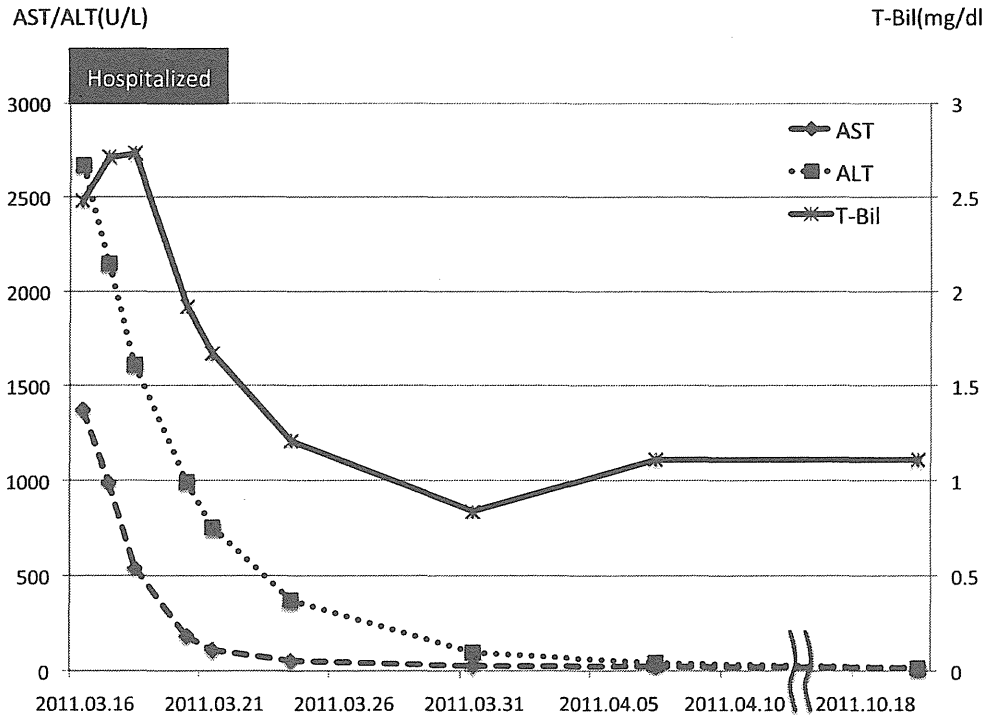

Fig. 1　Clinical course of the present case.

1）社会保険下関厚生病院消化器内科
2）東芝病院研究部
*Corresponding author: kl.okita@kousei-h.jp
＜受付日2012年3月8日＞＜採択日2012年6月30日＞

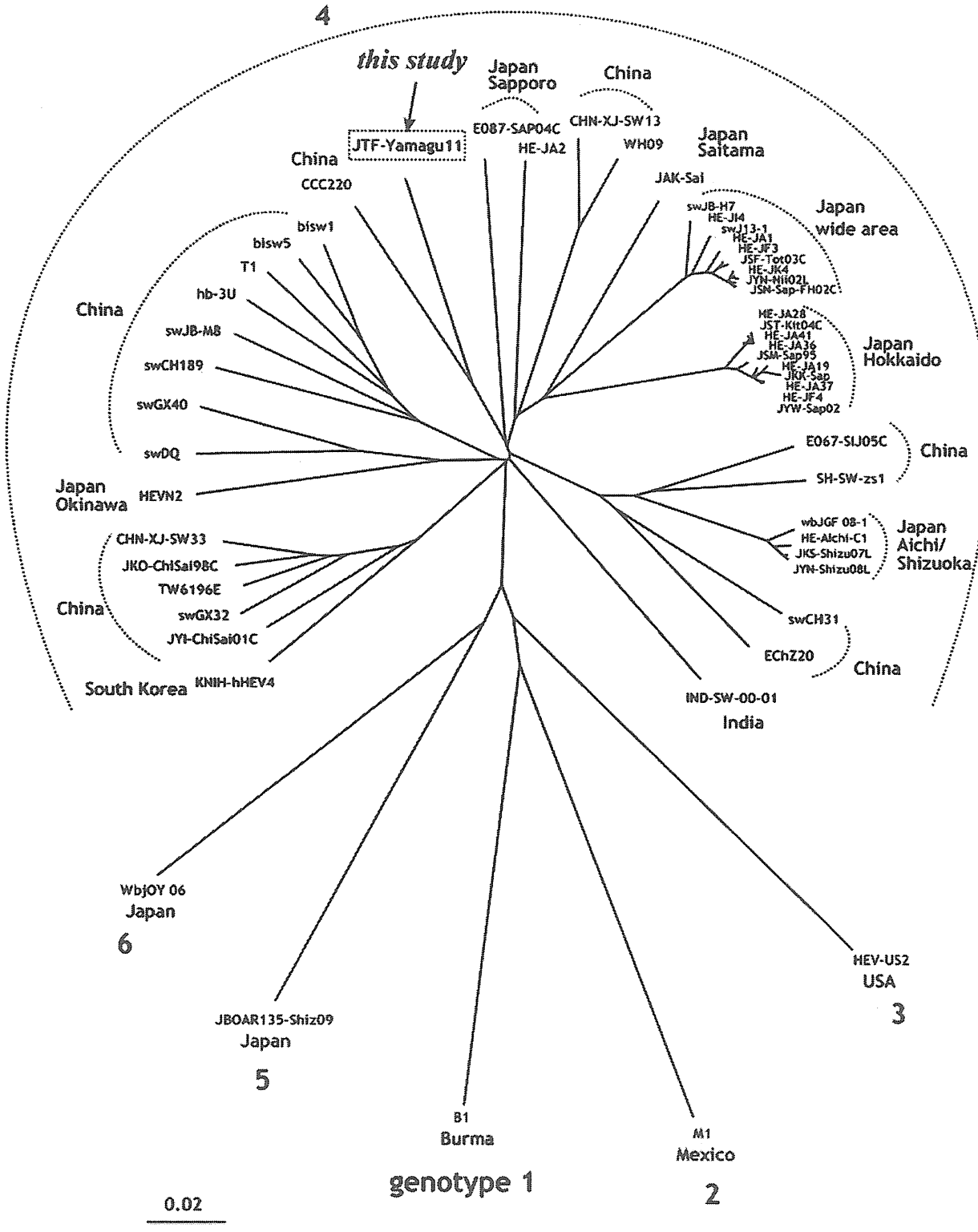

Fig. 2 Phylogenetic tree of HEV based on nearly full-genome nucleotide sequences. JTF-Yamagu11 (accession number AB698654) separated from the present case showed highest homology to CCC220 (AB108537) from Changchun, China.

その約1カ月後より全身倦怠感が出現し，近医受診．血液検査で肝機能異常を指摘され3月17日当院に紹介された．初診時の血液検査ではAST 1,371 IU/L，ALT 2,681 IU/L，総ビリルビン2.48 mg/dlと肝機能異常を認めたため，同日緊急入院とした．IgMクラスHA抗体，HBs抗原，IgM-HBc抗体，HCV抗体およびHCV-RNAは全て陰性であった．また，抗核抗体は40倍未満，抗ミトコンドリアM2抗体は5倍未満であり，腹部超音波検査でも肝腎コントラストは正常で，慢性肝障害を疑う所見もなかった．本人からの病歴聴取によりイノシシの肉と肝臓の摂取が判明し，E型肝炎検査を施行したところ，IgM-HEV抗体，IgA-HEV抗体とも陽性であり，かつHEV-RNAも検出されたことからE型急性肝炎と診断した．初診時の血液検査では，BUN 11.6 mg/dl，PT% 113.2%と異常なく劇症化の可能性も低いと判断した．安静加療としたところ，Fig. 1の如くトランスアミナーゼ，総ビリルビンとも速やかに改善し，自覚症状も消失し第7病日退院とした．以降も外来でフォローアップしたが肝機能の増悪なく半年後には基準値内に戻った．

本症例から分離したHEV-RNAについて塩基配列の完全長化を試み，7,236ntの準完全長塩基配列（JTF-Yamagu11，AB698654）が得られた．このHEV-RNAはgenotype 4型に属したが，既報の完全長および準完全長HEV塩基配列株と比較したところ，日本各地で分離同定されているgenotype 4型よりもむしろ中国東北部で採取された長春株(CCC220)[2]との間に比較的高い相同性を示した．分子系統樹解析においても山口株(JTF-Yamagu11）と長春株（CCC220）は近似性を示した(Fig. 2)．

考察：E型肝炎がイノシシ・シカ[3]・ブタ[4]などの肉や内臓を摂取し発症する人畜共通感染症であることはこれまでの報告から既に周知である．一般に，診断に至るまでの過程においてHEV流行地域への渡航歴と上述動物肉の摂取歴を聴取することは重要である．今回の症例では，当院紹介時点でイノシシの肉及び肝臓の摂取が判明しており，鑑別としてHEV感染をあげることは容易であり迅速な診断が可能であった．同時に摂食した家族3名に当科受診を強く勧めたが，本患者以外に自覚症状なく，一人のみ受診に終わった．その家族はIgG-HEV抗体は陽性であったが，IgM-HEV抗体，IgA-HEV抗体とも陰性であり，かつHEV-RNA検査でも陰性であった．初回の診察の際に本患者が一番多く摂取したと言われており，摂取量の多寡が感染の有無に関係したと示唆される．また，本例経験時にはまだ保険適応されていなかったIgA-HEV抗体の測定が，2011年秋に保険収載されたことから，今後本邦のE型肝炎の診断率が格段に向上すると期待される．

山口県からのE型急性肝炎の症例報告は今回で2例目である[5]．それに加え，我々の知る限りでは，山口県内で1999年以降に届け出がされたE型急性肝炎は自験例を含め全部で6例あった．Genotypeが判明しているものでは3型が1例，4型が2例である．一般的に北海道を除く日本各地ではGenotype 3が多いとされているが，北海道と離れている本県でこのような結果が出たことは興味深い．また，本症例のように準完全長塩基配列が解明できたのは山口県内で今回が初めてであり，しかも国内株よりも中国東北部（旧満州）由来のHEV株，特に長春株（CCC220）との近似性が高かったことは極めて興味深い．

山口県は地理的にも中国大陸に近く，有史以来，中国と交流があったことは確かである．人類史以前，中国大陸と日本がまた陸続きだった際にHEV感染した宿主が日本列島に流入，群れ同士が交配しないというイノシシの特性から中国株が温存された可能性も考えられる．一方で，ヒトと一緒にHEV感染した宿主が海を渡って，国内に入ってきてその後，野生化した可能性も否めない．阿部らの報告によれば[1]HEV感染患者のうち，58%の感染経路は不明である．既報の感染経路のみでは説明不可能なHEV感染症例が決して少なくないと考える．山口県に棲息する野生動物（特にイノシシ）に於けるHEV感染の実態解明が待たれる．

結語：山口県西部地区で狩猟されたイノシシが感染源であると考えられたE型急性肝炎の1例を経験し，本邦では未報告の中国長春株と近似するHEV塩基配列が得られたので報告した．

索引用語：E型肝炎，山口，イノシシ

文献：1）阿部敏紀，相川達也，赤羽賢浩，他．肝臓 2006；47：384—391　2）Liu Z, Chi B, Takahashi K, et al. Intervirology 2003; 46: 252—256　3）Tei S, Kitajima N, Takahashi K, et al. Lancet 2003; 362: 371—373　4）Yazaki Y, Mizuo H, Takahashi M, et al. J Gen Virol 2003; 84: 2351—2357　5）前田雅喜，浦田洋平，石川　剛，他．親子同時発症した急性E型肝炎．「急性肝不全」玄田拓哉，平野克治編，アークメディア，東京，2011，p125—126

英文要旨

A case of acute hepatitis E (genotype 4) after eating uncooked meat and liver of wild boar captured in Yamaguchi prefecture, with a viral genome relatively closer to a Chinese isolate than to Japanese strains

Kohsuke Okita[1]*, Kazuaki Takahashi[2], Katsunori Harada[1], Yukari Tanioka[1], Atsuyoshi Hirano[1], Teruaki Kimura[1], Akira Kato[1], Satoyoshi Yamashita[1], Masahiro Arai[2], Kiwamu Okita[1]

A 39 year-old male with liver dysfunction was admitted to our hospital and diagnosed of acute hepatitis because of detection of HEV-RNA in his serum. He had a history of eating uncooked meat and liver of wild boar captured in the western district of Yamaguchi prefecture, about one month before the onset of general fatigue. Analysis of HEV genome sequence revealed that the HEV isolated from the present case (JTF-Yamag11, AB698954) belonged to genotype 4 but showed poor homologies to Japanese genotype 4 strains so far reported. Instead and interestingly, however, it showed a certain relatedness to a Chinese isolate CCC 220 (AB108537) reported from Changchun, Jilin province (Liu et al. Intervirology 2003).

Key words: hepatitis E, Yamaguchi, wild boar

Kanzo 2012; 53: 534—537

1) Department of Gastroenterology and Hepatology, Social Insurance Shimonoseki Kousei Hospital
2) Department of Medical Sciences, Toshiba General Hospital
*Corresponding author: k1.okita@kousei-h.jp

J Gastroenterol (2013) 48:147–158
DOI 10.1007/s00535-012-0682-0

REVIEW

Culture systems for hepatitis E virus

Hiroaki Okamoto

Received: 6 July 2012 / Accepted: 31 August 2012 / Published online: 27 October 2012

Abstract The lack of an efficient cell culture system for hepatitis E virus (HEV) has greatly hampered detailed analyses of this virus. The first efficient cell culture systems for HEV that were developed were capable of secreting infectious HEV progenies in high titers into culture media, using PLC/PRF/5 cells derived from human hepatocellular carcinoma and A549 cells derived from human lung cancer as host cells. The success achieved with the original genotype 3 JE03-1760F strain has now been extended to various HEV strains in fecal and serum samples obtained from hepatitis E patients and to HEV strains in fecal and serum samples and liver tissues obtained from pigs and wild boar across species barriers. In addition, infectious HEV cDNA clones of the wild-type JE03-1760F strain and its variants have been engineered. Cell culture-generated HEV particles and those in circulating blood were found to be associated with lipids and open reading frame 3 (ORF3) protein, thereby likely contributing to the assembly and release of HEV from infected cells both in vivo and in vitro. The ORF3 protein interacts with the tumor susceptibility gene 101, a critical cellular protein required for the budding of enveloped viruses, through the Pro, Ser, Ala, and Pro (PSAP) motif in infected cells; ORF3 is co-localized with multivesicular bodies (MVBs) in the cytoplasm of infected cells, thus suggesting that HEV requires the MVB pathway for the egress of virus particles. This article reviews the development of efficient cell culture systems for a wide variety of infectious HEV strains obtained from humans, pigs, and wild boar, and also provides details of a new model for virion egress.

Keywords Hepatitis E virus · Cell culture · Infectious cDNA clone · Virion release

Abbreviations

CPE	Cytopathic effect
DN	Dominant negative
dpi	Days post-inoculation
ESCRT	Endosomal complex required for transport
HCV	Hepatitis C virus
HEV	Hepatitis E virus
HIV	Human immunodeficiency virus
kb	Kilobases
mAb	Monoclonal antibody
mutLSAL	A PASP mutant with two amino acid substitutions (Pro to Leu) in the PSAP motif of the ORF3 protein
MVB	Multivesicular body
ORFs	Open reading frames
RT-PCR	Reverse transcription-polymerase chain reaction
siRNA	Small interfering RNA
TGN	Trans-Golgi network
Tsg101	Tumor susceptibility gene 101
UTR	Untranslated region
Vps4	Vacuolar protein sorting-associated protein 4

Introduction

Hepatitis E virus (HEV) is the causative agent of acute or fulminant hepatitis, which occurs in many parts of the world, principally as a water-borne infection in developing

Part of this review was presented at The 3rd International Forum of the 98th General Meeting of the Japanese Society of Gastroenterology.

H. Okamoto (✉)
Division of Virology, Department of Infection and Immunity, Jichi Medical University School of Medicine,
3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan
e-mail: hokamoto@jichi.ac.jp

countries in Asia, Africa, and Latin America where sanitation conditions are suboptimal, and zoonotically in many industrialized countries including the United States, European countries, and Japan [1–6]. Hepatitis E has long been described as a self-limiting hepatitis that never progresses to chronicity [3]. However, since the identification of a case of chronic hepatitis E in Europe, chronicity has been documented in immunocompromised solid-organ transplant recipients and HIV-infected individuals [7–9].

HEV is classified in the genus *Hepevirus* within the family *Hepeviridae* [10]. The virion measures 27–34 nm in diameter and is believed to be non-enveloped, although HEV particles in circulating blood and culture supernatants are associated with lipids, as described in detail below. The HEV genome consists of a single-stranded, positive-sense RNA measuring approximately 7.2 kilobases (kb) in length, which is capped and polyadenylated [11, 12]. It contains a short 5′ untranslated region (UTR), three open reading frames (ORFs: ORF1, ORF2, and ORF3), and a 3′UTR. ORF1 encodes non-structural proteins including methyltransferase, papain-like cysteine protease, helicase, and RNA-dependent RNA polymerase [13, 14]. ORF2 and ORF3 overlap, and the ORF2 and ORF3 proteins are translated from a single bicistronic subgenomic RNA measuring 2.2-kb in length [15]. The ORF2 protein is the viral capsid protein, which works for particle assembly, binding to host cells, and eliciting neutralizing antibodies; the crystal structure of a truncated recombinant ORF2 protein has been elucidated [16, 17], but the structure of the whole capsid protein has not yet been resolved [18]. The ORF3 protein, which is a small phosphoprotein made of 113 or 114 amino acids (aa), is necessary for virion release [19, 20], as is described in detail below.

The discoveries of animal strains of HEV from domestic pigs, wild boar, deer, mongoose, rabbits, rats, bats, chickens, and fish (trout) have significantly broadened the host range and genomic diversity of HEV [5, 21–29], and the existence of at least four putative genera in the family *Hepeviridae*, one comprising human HEV genotypes and closely related animal viruses, and the other three including viruses from rodent (rat), chiropteran (bat), and avian (chicken) hosts, has been suggested [28]. The trout hepevirus might correspond to a separate taxonomic unit of higher rank, e.g., a subfamily [30]. Hepatitis E is now a recognized zoonotic disease, and several species of animals, such as swine, wild boar, and deer, are considered to serve as reservoirs of HEV strains that infect humans [31–36]. The ingestion of uncooked or undercooked meat and the viscera of farmed pigs and wild boar may be the major route of HEV infection in industrialized countries, including Japan [5, 6, 37]. Four major genotypes of HEV that infect humans have been identified [38, 39]. Genotypes 1 and 2 have so far been isolated only from humans and are mainly seen in developing countries. Genotypes 3 and 4 are zoonotic, and have been identified in many sporadic cases affecting middle-aged and elderly men in industrialized countries [4, 39–41]. Although two HEV strains (JBOAR135-Shiz09 and wbJOY_06) of new unrecognized genotypes, provisionally designated genotypes 5 and 6, respectively, that cluster closely with human HEV strains but are markedly divergent from rat, bat, and avian HEV strains, have been detected from wild boar in Japan, it remains unknown whether these boar strains can infect humans across species [42, 43].

HEV has been regarded as a virus that is impossible to culture in vitro, given the tremendous difficulties in developing a cell culture system. There are no small animal models of infection, and macaques are inaccessible or unaffordable for most researchers in the field. The establishment of a practical cell culture system that facilitates the propagation of HEV in vitro is critical for virological characterization, as well as for studies on the prevention of HEV infection. This article reviews the breakthrough in the establishment of cell culture systems for various HEV strains from humans, pigs, and wild boar, and provides details of a new model for virion egress.

Establishment of cell culture systems for HEV strains of genotypes 3 and 4 found in feces and serial passages of their progenies in cultured cells

HEV usually replicates to low titers in vivo, and growing it in cell culture has proven to be exceedingly difficult. However, a breakthrough has been achieved recently using a fecal specimen from a sporadic case of acute hepatitis E in Japan that contained a very high load of HEV (JE03-1760F strain of genotype 3: 2.0×10^7 copies/ml), which thus enabled the establishment of a robust culture system for HEV. The fecal suspension was used as an inoculum, and the replication capacity of HEV was then 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). The JE03-1760F strain was found to replicate efficiently in two cell lines; PLC/PRF/5 (Alexander) and A549 from human lung cancer [44]. Of note, although A549 cells are not hepatocytes, HEV was shown to be distributed not only in the liver, but also in the lung, kidney, spleen, and even colon in infected pigs [45]. The presence of HEV in bile, lymph nodes, and tonsils in infected pigs has also been reported [46, 47].

Upon inoculation of the JE03-1760F strain, progeny viruses were excreted into the culture media in titers reflective of the HEV inoculum. HEV grew efficiently following the seeding of 1.0×10^5 copies (genome copies per cells: approximately 0.1) in the wells of a six-well microplate containing monolayers of PLC/PRF/5 or A549 cells, and the viral load in the culture medium reached

10^8 or 10^7 copies/ml, respectively, on day 50. The first successful propagation of the JE03-1760F strain in PLC/PRF/5 and A549 cells may have been ascribable to the markedly high HEV titer in the inoculum. RNA viruses generally exist as quasispecies [48]. A sample with a high titer has an increased probability of containing variant(s) with advantageous mutations needed to permit the infection of a cultured cell. Notably, the JE03-1760F strain harbored the 29 point mutations with 6 non-synonymous mutations found in the virus in feces, which were not possessed by any of the reported genotype 3 HEV strains [49].

The initial success was followed by the establishment of a culture system with the use of another fecal suspension, from a patient with fulminant hepatitis E, containing the HE-JF5/15F strain of genotype 4 at a high titer (1.3×10^7 copies/ml) [50]. The HE-JF5/15F strain was propagated and passaged more efficiently than the JE03-1760F strain [51]. Epidemiological surveys suggest that HEV of genotype 4 is significantly more frequently associated with a severe form of hepatitis than HEV of genotype 3 [52–54]. The high replicative activity of HEV genotype 4, reproduced in the culture system for the HE-JF5/15F strain of this genotype, is expected to be useful for elucidating the viral factors associated with the development of fulminant hepatitis E in infected patients.

Furthermore, HEV progenies of the genotype 3 JE03-1760F strain in the culture supernatant grew efficiently through many generations of passages in PLC/PRF/5 and A549 cells (53 generations of passages as of June 2012), with the highest HEV RNA titer in the culture media being 10^8–10^9 copies/ml (unpublished observations). In addition, HEV progenies of the genotype 4 HE-JF5/15F strain in the culture supernatant replicated efficiently through many generations of passages in PLC/PRF/5 and A549 cells (33 generations of passages as of June 2012), with the highest HEV loads in the culture supernatants being 10^9–10^{10} copies/ml (unpublished observations). Shukla et al. [55] described the adaptation of the Kernow C1 strain, a genotype 3 HEV strain purified from the feces of a chronically infected patient, to growth in human hepatoma cells (HepG2/C3A) selected for a virus recombinant that contained an insertion of 174 ribonucleotides (58 amino acids) of a human ribosomal protein gene. However, no such insertion or deletion of nucleotides was observed in the genomes of the cell-culture adapted JE03-1760F and HE-JF5/15F strains during the long-term passages. No cytopathic effect (CPE) was observed in the PLC/PRF/5 and A549 cells during these serial passages of the JE03-1760F and HE-JF5/15F strains, despite differences in the durations of the cell culture and the changing profile of the HEV load in each passage.

Although HepG2 and Huh7 cells are not permissive for the wild-type JE03-1760F and HE-JF5/15F strains of feces origin, recent studies have demonstrated that both these cell lines are capable of supporting the successful propagation and passages of cell-culture adapted JE03-1760F and HE-JF5/15F strains (unpublished observations).

Propagation of HEV strains found in circulating blood in cultured cells

HEV is an emerging infectious threat to blood safety. There have been a number of publications delineating this threat by providing evidence of the transmissibility of this virus through transfusions. Transfusion-transmitted HEV infection has been reported not only in developing countries (genotype 1) [56], but also in industrialized countries, including Japan and the United Kingdom (genotypes 3 and 4) [57–60], thus suggesting that HEV in circulating blood can also grow in cultured cells. Therefore, this study examined whether HEV strains in serum samples could replicate in PLC/PRF/5 and A549 cells and release infectious progeny viruses into the culture media; this was investigated in relation to the HEV load, genotype, and co-existence of HEV antibodies [61]. The study revealed that various HEV strains of genotype 1, 3, or 4 in the serum samples obtained from patients with autochthonous or imported hepatitis E could infect and replicate efficiently in PLC/PRF/5 and A549 cells. Notably, HEV strains in all serum samples tested, with or without concurrent HEV antibodies, were successfully propagated in cultured cells when inoculated at an HEV load of $\geq 10^5$ copies per well in a six-well microplate (Fig. 1). Progeny viruses of serum-derived HEV strains in the culture supernatant were successfully passaged in PLC/PRF/5 and A549 cells, thus indicating that the HEV progenies of serum origin released from cultured cells were infectious, similar to those of feces origin [61].

Characterization of HEV particles in culture supernatant, circulating blood, and feces

An examination of the physicochemical properties of HEV particles in the culture supernatant revealed the HEV particles in cell culture to have a buoyant density of 1.15–1.16 g/ml in sucrose gradients [62], which was identical to the finding in serum samples, irrespective of the presence or absence of circulating anti-HEV antibodies, but markedly lower than that in feces, which peaked at 1.27–1.28 g/ml [61] (Table 1). The great majority (more than 90 %) of HEV particles in the circulation were free of immunoglobulins even in the presence of IgM anti-HEV antibodies [61]. Similar to cell culture-generated HEV particles, HEV particles in serum were non-neutralizable

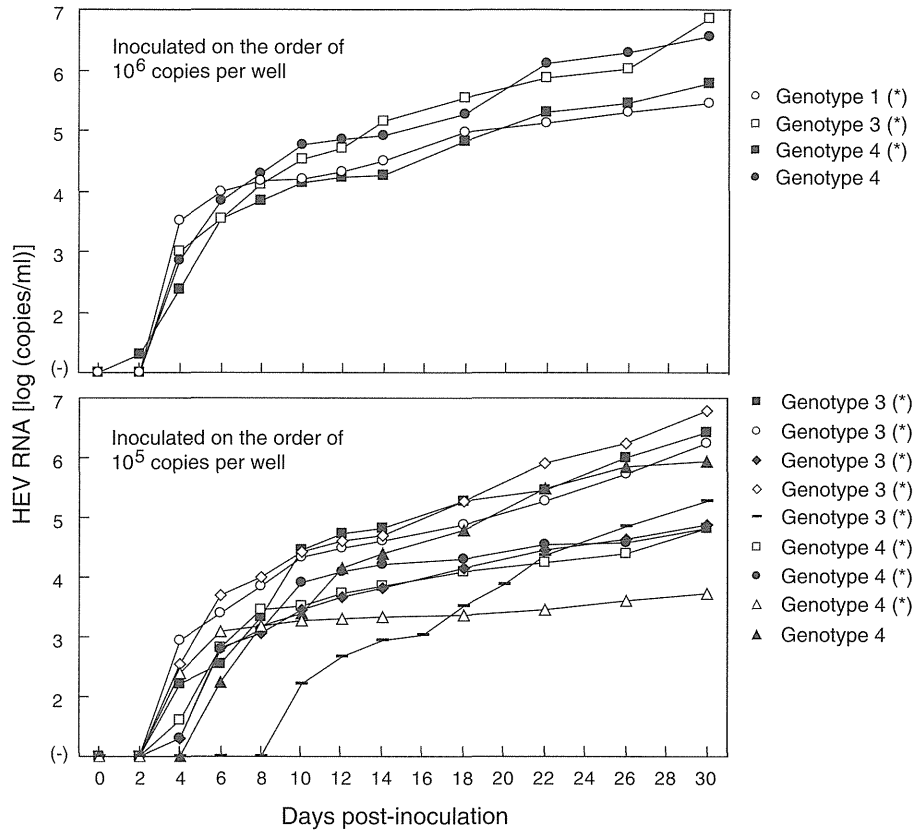

Fig. 1 Quantification of hepatitis E virus (HEV) RNA in culture supernatants of PLC/PRF/5 cells after inoculation of serum samples at a viral load on the order of 10^6 (*upper panel*) or 10^5 (*lower panel*) copies per well, containing HEV of the indicated genotype. The *asterisks in parentheses* indicate serum samples with anti-HEV antibodies. See reference [61] for further details

by immune sera and anti-ORF2 monoclonal antibodies (mAbs) that could definitely neutralize the infection of HEV in feces in the cell culture system [44, 62], and few or no virus particles in either the serum or cell culture were captured by anti-ORF2 mAb and anti-ORF3 mAb. Interestingly, however, after treatment with a detergent such as deoxycholic acid, Tween 20, or NP-40, the binding efficiency of HEV particles in serum and culture supernatant to both anti-ORF2 and anti-ORF3 mAbs markedly increased [61], thus suggesting that the HEV virion in both the serum and culture medium possesses the ORF3 protein on its surface, in association with lipids [19].

The detergent-treated HEV virions in the serum and culture medium were partially neutralized by anti-HEV antibodies, which may be ascribable to the incomplete exposure of ORF2 and ORF3 proteins after treatment with detergent only. The cell culture-produced and serum HEV particles treated with both detergent and protease, with a buoyant density of 1.27-1.28 g/ml in sucrose (Table 1), can be neutralized by an anti-HEV immune serum and anti-ORF2 mAb, thus indicating that virions treated with lipid solvent and proteases, possessing the same characteristics as virions from feces, are neutralizable by anti-HEV immune sera. The ORF3 protein is required for virion release from cultured cells. Taken together, these findings indicate that it is very likely that HEV particles are released from both infected cultured cells (in vitro) and infected hepatocytes (in vivo) as lipid-associated virions, accompanied by ORF3 protein, and that the ORF3 protein and lipids are dissociated from the virion after shedding in the bile duct, which contain detergent (deoxycholic acid), and then in the duodenum, which contain protease (trypsin) secreted from the pancreas (Fig. 2).

HEV attaches to the host cell via a specific high-affinity receptor and enters the cytoplasm by clathrin-mediated endocytosis [63]. However, virtually nothing is known about the mechanism by which HEV enters susceptible cells. It is interesting that non-neutralizable HEV particles in serum samples and culture supernatant, which are associated with lipids and band at 1.15-1.16 g/ml in sucrose gradients, can bind to cultured cells, although inefficiently, and can be propagated in cultured cells [19]. Furthermore, a lipid solvent did not abolish or increase the infectivity of the HEV progenies obtained from infected cells, and both the cell culture-generated HEV particles and those that were treated with detergent and/or protease were propagated in the cultured cells with nearly identical efficiency [61], regardless of the presence or absence of the ORF3 protein and lipids on the surface. These findings, taken together, suggest that it is unlikely that only a fraction of HEV particles in the serum samples and culture media, partially unaccompanied by lipids, can bind to the

Table 1 Changes in the buoyant density and antigenicity in hepatitis E virus (HEV) particles of distinct origin after treatment with deoxycholic acid and/or trypsin

HEV particles	Treatment		
	None	Deoxycholic acid[a]	Deoxycholic acid + trypsin[a]
Culture supernatant			
Density in sucrose (g/ml)	1.15–1.16	1.21–1.24	1.27–1.28
Detectability by			
Anti-ORF2 mAb	No	Yes	Yes
Anti-ORF3 mAb	No	Yes	No
Neutralizability by			
Immune sera	No	Yes	Yes
Anti-ORF2 mAb	No	Yes	Yes
Serum			
Density in sucrose (g/ml)	1.15–1.16	1.21–1.24	1.27–1.28
Detectable by			
Anti-ORF2 mAb	No	Yes	Yes
Anti-ORF3 mAb	No	Yes	No
Neutralizability by			
Immune sera	No	Yes	Yes
Anti-ORF2 mAb	No	Yes	Yes
Feces			
Density in sucrose (g/ml)	1.27–1.28	1.27–1.28	1.27–1.28
Detectability by			
Anti-ORF2 mAb	Yes	Yes	Yes
Anti-ORF3 mAb	No	No	No
Neutralizability by			
Immune sera	Yes	Yes	Yes
Anti-ORF2 mAb	Yes	Yes	Yes

ORF2 open reading frame 2, *mAb* monoclonal antibody

[a] Treated with 0.1 % deoxycholic acid and/or 0.1 % trypsin

cell surface receptors and enter susceptible cells. In order to provide a plausible explanation(s) for this intriguing observed phenomenon, future studies must therefore elucidate whether and how non-neutralizable HEV particles in the serum and culture supernatant can enter susceptible cells.

Propagation of HEV strains in cell culture across species barrier

Shukla et al. [55] recently showed that genotype 3 viruses infected swine cells (LLC-PK1) more efficiently than human cells (HepG2/C3A). A549 cells can also support the replication of swine HEV of genotype 4 in a fecal specimen [64]. However, it remains unclear whether swine HEV strains of genotypes 3 and 4 recovered from domestic pigs and wild boar can grow as efficiently as human HEV strains in human cultured cells, irrespective of the source of the inoculum virus. Therefore, to investigate this problem, liver homogenate, serum and fecal specimens obtained from HEV-infected domestic pigs and wild boar were employed as inocula, and various swine and boar HEV strains were cultivated in A549 and PLC/PRF/5 cells and their replication efficiency was evaluated in relation to the viral load and origin of the inoculum [65].

Inoculation of three HEV RNA-positive liver homogenate samples with a higher HEV load (4.0×10^4–6.6×10^5 copies per well) obtained from raw pig liver, sold as food that had been purchased from grocery stores and kept frozen at −80 °C [6], released HEV progeny viruses into the culture medium. Figure 3a shows that HEV RNA was first detected in the culture medium of A549 cells on the 2nd to 6th day post-inoculation (dpi), with a viral load of 40–220 copies/ml, and continued to increase thereafter, with the highest titer of 1.1–7.3×10^7 copies/ml on 30 dpi. HEV progenies were also released into the culture medium upon the inoculation of fecal and serum samples [65].

Efficient viral multiplication was observed upon the inoculation of four liver homogenate samples containing boar HEV at an HEV load of 9.8×10^5–6.6×10^6 copies per well [65]. HEV RNA was initially detected on day 2 and reached the highest titer of 9.8×10^7–3.5×10^8 copies/ml on day 50 (Fig. 3b). Overall, HEV progenies were released into the culture medium when swine and boar

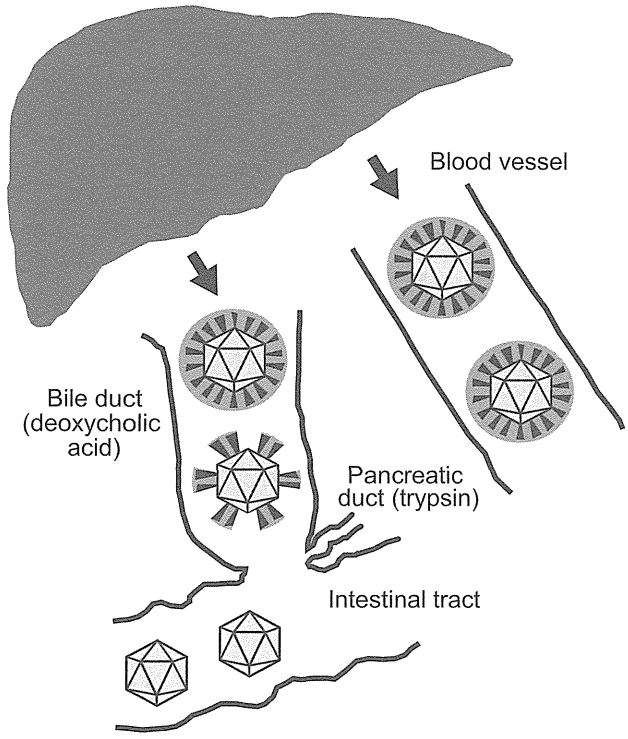

Fig. 2 Schematic diagram of the "enveloped" and "non-enveloped" HEV particles in infected hosts. HEV particles excreted from the liver into the circulation are likely to be present as "enveloped" virus with cellular membranes and open reading frame 3 (ORF3) proteins on their surfaces, while the ORF3 protein and cellular membranes are dissociated from the virion after shedding in the bile duct, which contains detergent (deoxycholic acid) and then in the duodenum, which contains protease (trypsin), both secreted from the pancreas

HEV strains were inoculated at an HEV load of $\geq 2.0 \times 10^4$ copies per well, irrespective of the inoculum source, although the HEV RNA titers in the culture medium of 30 dpi differed markedly, ranging from 1.0×10^2 to 1.1×10^7 copies/ml, by inoculum, when inoculated at viral loads ranging from 2.0×10^4 to 1.1×10^5 copies per well [65]. In sharp contrast, no progenies were detectable in the culture supernatant upon the inoculation of swine and boar HEV strains at an HEV load of $<1.8 \times 10^4$ copies per well. These results indicate that swine HEV strains of genotypes 3 and 4 from domestic pigs and wild boar can replicate as efficiently as human HEV strains in human cultured cells, thereby supporting the zoonotic nature of HEV in the culture systems for HEV as well.

Construction of infectious cDNA clones of wild-type and mutated HEV genomes and analysis of the function of the ORF3 protein in virion egress

A full-length infectious cDNA clone (pJE03-1760F/wt) of the genotype 3 JE03-1760F strain was developed [66]. The inoculation of the pJE03-1760F/wild-type (wt) virus into PLC/PRF/5 or A549 cells in six-well plates at 1.0×10^5 copies per well allowed the cDNA-derived virus to grow as efficiently as the original feces-derived virus in both PLC/PRF/5 and A549 cells, thus reaching 10^6 copies/ml on 30 dpi in the respective cells. Therefore, the reverse genetics system for HEV is utilizable in a robust cell culture system to elucidate the mechanism of HEV replication and the functional roles of HEV proteins.

Previous studies suggested the expression of the intact ORF3 protein to be essential for the infection of animals [67, 68], but this protein is not required for infection and virion morphogenesis in vitro [69]. However, an immunocapture reverse transcription-polymerase chain reaction (RT-PCR) assay using an anti-ORF3 mAb suggested the presence of ORF3 protein on the surfaces of cell culture-generated HEV particles and those in circulating blood [61, 70]. Therefore, to examine whether ORF3 protein is responsible for virion morphogenesis and viral release from infected cells [19], a full-length cDNA clone of an ORF3-deficient mutant (pJE03-1760F/ΔORF3; hereafter, ΔORF3 for simplicity) was constructed by mutating the initiation codon of the ORF3 gene (ATG to GCA) on the pJE03-1760F/wt cDNA clone (Fig. 4a).

The culture supernatant of cells transfected with RNA transcripts of the ΔORF3 cDNA clone was inoculated onto A549 cells to determine whether the ΔORF3 mutant virus in the culture medium of the transfected cells was infectious and replication-competent and, if so, whether HEV virions were released from the ΔORF3 mutant-infected cultured cells. Viral RNA was nearly undetectable in the culture supernatant of A549 cells inoculated with the ΔORF3 mutant (Fig. 4b). Despite the significantly lower levels of HEV RNA in the culture supernatant of ΔORF3 mutant-inoculated A549 cells, the intracellular HEV RNA was nearly equal to that observed in cultured cells inoculated with the pJE03-1760F/wt (Fig. 4b). When applied to sucrose density ultracentrifugation, the ΔORF3 mutant in the culture supernatant of the transfected cells banded at 1.27–1.28 g/ml, in contrast to the pJE03-1760F/wt virus, which peaked at 1.15–1.16 g/ml (Fig. 4c) [19].

The advent of an infectious cDNA clone of genotype 1 HEV (the Sar55 strain), allowed Emerson et al. [20] to confirm the findings of the previous study conducted by using a genotype 3 infectious cDNA clone of the JE03-1760F strain, which reported the ORF3 protein to be essential for virion egress from infected cells, the ORF3 protein to be present on the surfaces of HEV particles released from infected cells, and the HEV particles released from infected cells to be lipid-associated [19]. Genotypes 2 and 4 HEVs have not yet been examined for the function of the ORF3 protein in virion egress using infectious cDNA clones. However, given its conservation,

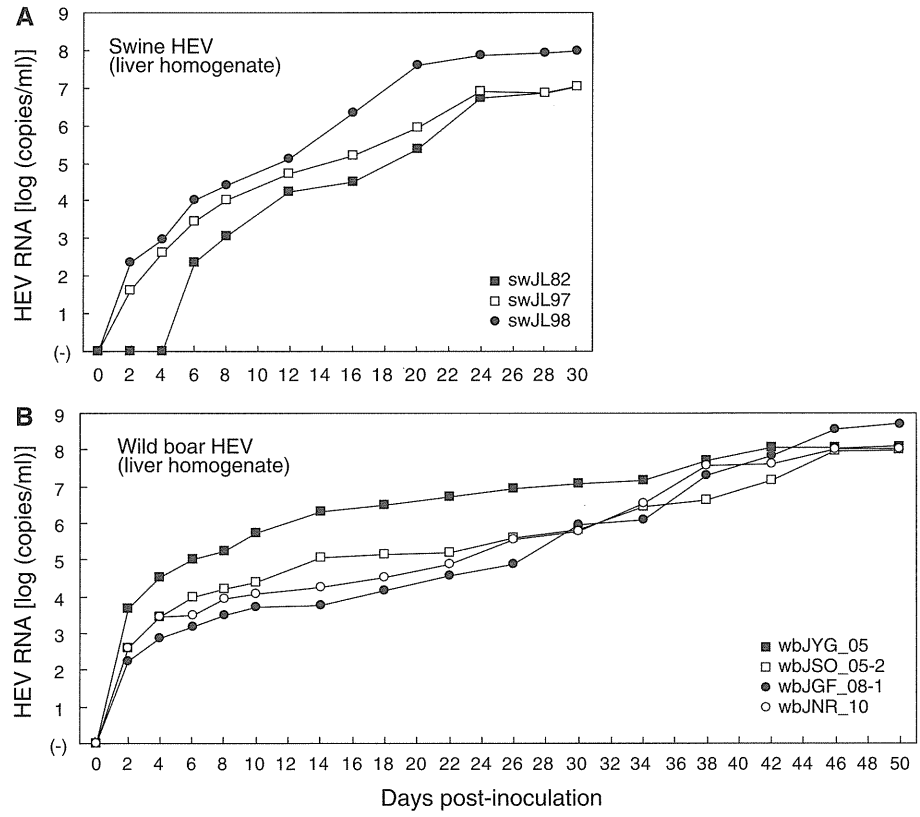

Fig. 3 Quantification of HEV RNA in the culture supernatants of A549 cells inoculated with homogenate samples of swine (*sw*) liver tissues (**a**) and wild boar (*wb*) liver tissues (**b**). Modified from reference [65]

and because the association of HEV virions of genotype 1, 3, or 4 in the blood circulation with ORF3 protein and lipids on the surface has been noted [61], it is very likely that the function of the ORF3 protein related to HEV morphogenesis is common to all HEV strains, irrespective of genotype.

A Pro-rich sequence is present in the C-terminal region of the ORF3 protein in all mammalian and avian HEV strains, and the Pro, Ser, Ala, and Pro (PSAP) motif between amino acid residues 95 and 98 of the ORF3 protein (Fig. 4a) is conserved among all known HEV strains, including avian HEV strains [71]. The introduction of amino acid substitutions (two Pro-to-Leu replacements) in the PSAP motif (mutLSAL) (Fig. 4a) significantly reduced the virus yield in the culture supernatant, similar to an ORF3-deficient variant, ΔORF3 (Fig. 4b). Notably, no significant difference in the intracellular HEV RNA level was observed upon inoculation of the wild-type virus and its ORF3 variants (Fig. 4b). The particles generated in the culture supernatant of the mutLSAL RNA-transfected cells banded at 1.26–1.27 g/ml, similar to the particles in the ΔORF3 RNA-transfected cells (Fig. 4c), thus suggesting that the PSAP motif in the ORF3 protein is indispensable for the formation of membrane-associated HEV particles. Viral particles in the culture supernatant of the mutLSAL RNA-transfected cells were efficiently captured by an anti-ORF2 mAb, but not by an anti-ORF3 mAb, which was similar to the findings observed in the supernatant of the ΔORF3 RNA-transfected cells, with or without prior treatment with 0.1 % sodium deoxycholic acid. These results indicate that an intact PSAP motif in the ORF3 protein plays a pivotal role in the release of HEV particles having lipid-associated membranes and ORF3 protein [71].

Cellular factors involved in the release of hepatitis E virions

Recent studies have revealed that viral matrix proteins play critical roles during the later stages of virus budding in many enveloped RNA viruses, including retroviruses, orthomyxoviruses, rhabdoviruses, and filoviruses: these viral proteins possess a so-called late (L)-domain containing P(T/S)AP, PPxY, and YxxL, which are critical motifs for the efficient release of an enveloped virus [72–76], and they hijack host proteins in the vacuolar protein sorting pathway [75, 77]. This pathway gives rise to multivesicular bodies (MVBs), which are topologically identical to virus budding [78]. The PTAP motif was first identified in the Gag protein of human immunodeficiency virus (HIV) and has been reported to bind to the tumor susceptibility gene 101 (Tsg101), which is identified as a critical cellular protein required for the budding of enveloped viruses, e.g., HIV and Ebola virus, from the plasma membrane [79, 80].

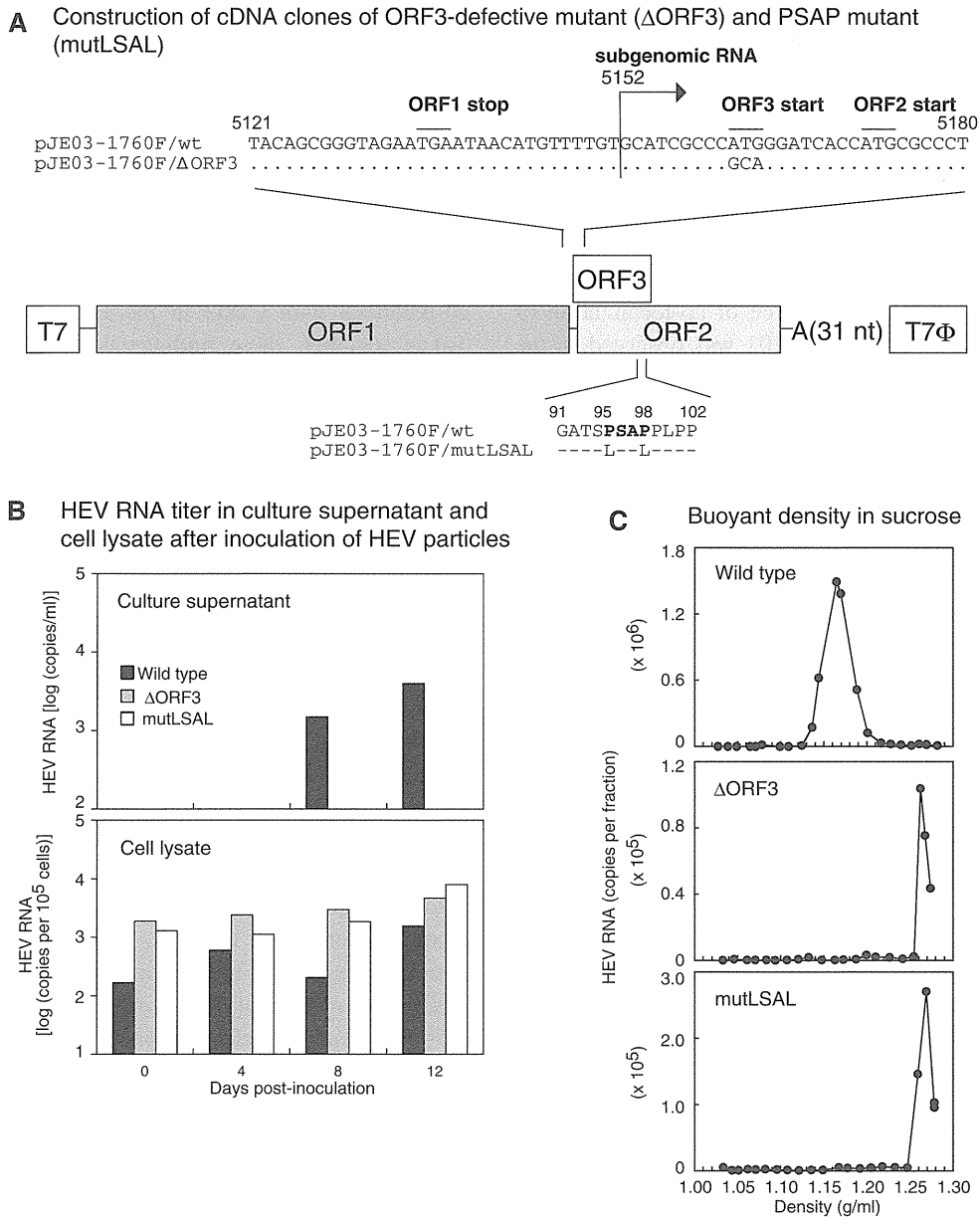

Fig. 4 Characterization of the ORF3 mutants of HEV. **a** Schematic illustration of the full-length cDNA clone of the HEV JE03-1760F strain (pJE03-1760F/wild-type [wt]) and its derivative mutants. The nucleotide (*nt*) sequence of nt 5121–5180 of the full-length cDNA clones of pJE03-1760F/wt and pJE03-1760F/ΔORF3 (ΔORF3, for simplicity) and the amino acid sequence of amino acid (aa) 91–102 of the ORF3 protein of the full-length cDNA clones of pJE03-1760F/wt and its Pro, Ser, Ala, and Pro (*PSAP*) mutant mutLSAL (pJE03-1760F/mutLSAL; mutLSAL, for simplicity) are aligned. Although not illustrated in the Fig., a Ser-to-Leu mutation at aa 87, which is identical to the majority of reported genotype 3 HEV strains, was also introduced in the mutLSAL. The stop codon of the ORF1 gene and the proposed initiation codons of the ORF2 and ORF3 genes are indicated by *lines above the nucleotides*. The *dots* represent nucleotides identical to those at the top (wild-type). The initiation site of subgenomic mRNA transcription [92] is depicted by a *vertical line with an arrow facing right*. The ΔORF3 mutant was generated by mutating ATG (Met) to GCA (Ala) at the start codon of the ORF3 gene. The mutLSAL mutant was produced by converting CCC (Pro) to CTC (Leu) at the 95th codon and CCT (Pro) to CTT (Leu) at the 98th codon of the ORF3 gene. **b** HEV RNA in the culture supernatant (*upper panel*) and cell lysate (*lower panel*) of A549 cells inoculated with the culture supernatant of wild-type, ΔORF3, or mutLSAL RNA-transfected PLC/PRF/5 cells was quantified by measuring the RNA titer by real-time reverse transcription-polymerase chain reaction (RT-PCR). **c** Sucrose density-gradient fractionation of HEV in culture supernatants from PLC/PRF/5 cells transfected with RNA transcripts of wild-type, ΔORF3, or mutLSAL. Modified from reference [71]

Tsg101 binds to the PSAP motif of HEV located within the ORF3 protein [81]. A co-immunoprecipitation procedure also showed the direct interaction of ORF3 protein with Tsg101 and the loss thereof with the PSAP mutations in PLC/PRF/5 cells transfected with a full-length wild-type or mutant ORF3 expression plasmid [71, 82]. These results

indicate the requirement of an intact PSAP motif in the ORF3 protein for the formation and release of membrane-associated HEV particles possessing ORF3 proteins on the surface, in agreement with the observation by Emerson et al. [20], who reported the PxxP motif of the ORF3 protein to play an important role in virus egress and infection.

When the intracellular localization of the ORF3 protein and Tsg101 was examined by immunofluorescence confocal microscopy, a high degree of co-localization of wild-type ORF3 protein and Tsg101 was observed in the cytoplasm. In contrast, the mutLSAL ORF3 protein showed essentially no signal of co-localization with Tsg101, despite the fact that the mutated ORF3 protein showed an intracellular localization similar to that of the wild-type ORF3 protein. These results support the notion that the ORF3 protein interacts with Tsg101 through its PSAP motif in infected cells [82]. The effect on virion release from infected cells was examined by utilizing small interfering (si) RNA against Tsg101 to examine whether Tsg101 was functionally involved in HEV budding through its interaction with the ORF3 protein. The depletion of endogenous Tsg101 by siRNA led to a significant reduction of HEV release in cultured cells, although the HEV RNA replication was not affected by the transfection of siRNA against Tsg101. These results indicate that Tsg101 plays a pivotal role specifically in the release of HEV virions, thus corroborating the role attributed to Tsg101 in the budding of other known enveloped viruses such as retroviruses [80], although HEV is known to be a non-enveloped virus. It was also strongly suggested that HEV utilizes the mechanism of cellular MVB sorting, because Tsg101 is a component of the endosomal complex required for transport (ESCRT)-I complex which is involved in MVB sorting.

The vacuolar protein sorting-associated protein 4 (Vps4) ATPase is one of the final effectors in the MVB pathway and functions downstream of Tsg101, and therefore it was hypothesized that dominant negative (DN) mutants of Vps4 might disrupt virion release. In fact, DN mutants of Vps4 inhibit the budding of infectious HIV particles in co-transfection experiments [76]. The overexpression of DN mutants of Vps4 inhibited the release of HEV substantially. The reduction in the release of HEV particles in the presence of DN mutants of Vps4 appears to be due to a specific block in the formation of membrane-associated virions. It is likely that Vps4 is required for the final step in the formation of membrane-associated HEV particles. These results suggest that HEV utilizes the MVB pathway for virion egress, and that the enzymatic activities of Vps4 are involved in the virus release [82].

Whether the membrane-associated HEV particles are generated intracellularly or at the cell surface remains unclear. HEV particles with lipid membranes and the ORF3 protein on their surfaces were found abundantly in the lysates of cells infected with wild-type HEV (unpublished observations), and the ORF3 protein and Tsg101 were co-localized in the cytoplasm, as described above, thus suggesting that mature membrane-associated HEV particles are generated before their release from the surfaces of infected cells. An immunofluorescence assay using anti-ORF3 mAb and antibody against CD63, another MVB marker protein, revealed that the ORF3 protein was co-localized with CD63 in the HEV-infected cells [82]. Taken together, these findings indicate that HEV likely utilizes the cellular ESCRT mechanism in the cytoplasm, but not at the cell surface, to induce the release from infected cells. Further studies are needed to clarify whether membrane-associated HEV particles really bud into the intracellular vesicles formed in infected cells, and whether they are derived from the Golgi apparatus, trans-Golgi network (TGN), or endosomes, and also whether the mature virions are released into the extracellular environment by the exosomal secretion pathway, similar to the pathway in known enveloped viruses such as the hepatitis C virus (HCV) [83, 84].

Although it has been reported that monotherapy using interferon or ribavirin inhibits the viral replication in immunocompromised patients with chronic HEV infection following solid-organ transplantation [85, 86], no approved antiviral drugs are yet available to prevent or treat HEV-associated diseases. A conserved virus release mechanism would therefore be likely to be an excellent target for antiviral drugs, and human tetherin/BST-2 is reported to inhibit the release of HIV, filoviruses, and herpesviruses [87–91]. It may therefore be interesting to analyze whether tetherin/BST-2 inhibits the virion release of HEV. A better understanding of the mechanisms whereby viruses recruit cellular factors should therefore help greatly in the development of new therapeutic strategies.

Conclusion

This article has described the development of the first efficient cell culture systems for a wide variety of HEV strains from humans, pigs, and wild boar. The unique characteristics of cell-culture-generated HEV particles and those in the circulating blood were reviewed, and the current knowledge on virion egress obtained by the established cell culture models, reinforced by a reverse genetics system, was introduced. These cell culture systems, with the aid of reverse genetics systems, will solve many mysteries and answer numerous questions surrounding the epidemiology of HEV infection, and will be useful for the further understanding of the HEV life cycle and the investigation of potential antiviral drugs, as well as