BRIEF REPORT

Intranasal Transmission of Hepatitis C Virus: Virological and Clinical Evidence

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Intranasal transmission of hepatitis C virus (HCV) via contaminated drug-sniffing implements is a potential but unconfirmed source of viral infection. We demonstrate the virological plausibility of intranasal transmission by confirming that blood and HCV RNA are present in the nasal secretions and drug-sniffing implements of HCV-infected intranasal drug users recruited from a community health clinic in New York City.

Hepatitis C virus (HCV) is the most common bloodborne pathogen in the United States and is a major cause of liver-related morbidity, mortality, and liver transplantation [1]. HCV is transmitted through contact with infected blood [2] (mostly via shared needles and other drug injection paraphernalia); however, a large proportion (up to 20%) of HCV infections remain unexplained, especially among noninjection drug users [3]. One hypothesis to account for these unexplained cases involves intranasal transmission of HCV via contaminated implements, such as straws, used to snort cocaine, heroin, and other powdered drugs [4]. Implements inserted into the nasal cavity, which has been eroded by long-term drug sniffing, might come into contact with HCV-infected mucus or blood, which might then be transmitted to a susceptible individual sharing the same implement [5]. Epidemiological studies of intranasal transmission of HCV have produced inconsistent findings [6, 7], in part because of the high correlation between drug sniffing and other risk factors for HCV infection. Here, we attempt to refute the intranasal transmission hypothesis by invalidating ≥1 of its virological preconditions. Specifically, we address 2 primary research questions: (1) Does HCV RNA exist in the nasal secretions of serum-positive drug sniffers? (2) If so, can HCV RNA be transferred onto the sniffing implements shared by intranasal drug users. A secondary aim was to examine clinical nasal pathologies that might facilitate intranasal HCV transmission.

Methods. Our sample included low-income, urban intranasal drug users with chronic, active HCV infection. Subjects were primarily Hispanic and African American and were recruited from a neighborhood health clinic in East Harlem, New York City, an area with a high prevalence of HCV infection (up to 29%) among noninjection drug users [3]. Eligibility criteria included (1) age, ≥18 years; (2) self-reported intranasal drug use; and (3) a positive result of a quantitative HCV PCR blood test. Overall, 38 patients enrolled in the study and provided informed consent. Study protocols were approved by 3 institutional review boards.

The following medical information was obtained from subjects: quantitative HCV RNA test result and viral load, hepatitis B antibody test results, liver enzyme levels (i.e., alanine aminotransferase level), and liver biopsy history. Subjects completed a brief survey, in either Spanish or English, that covered demographic characteristics, risk factors for HCV infection, injection and noninjection drug use, health status, and nasal pathology symptoms.

Blood samples were collected for quantitative PCR. Two nasal secretion samples (1 from each nostril) were collected with Dacron nasal swabs and placed in (1) 1 mL of TRIzol reagent (Gibco BRL) for RNA detection or (2) 1 mL of OBTI solution for blood detection. Similarly, 2 experimental sniffing implements, which consisted of new (packaged) soda straws commonly used by drug sniffers, were collected from each subject. To avoid harmful effects of sniffing powdered substances, subjects were instructed to "snort air" while mimicking their normal drug-sniffing behavior.

HCV RNA was isolated from 200 μL of serum by use of the QIAamp MinElute kit (Qiagen); HCV RNA was isolated from nasal secretions and sniffing implements using the TRIzol (Gibco BRL) on the basis of established protocols [8]. The first strand of cDNA was synthesized by ImProm-IITM Reverse Transcription System (Promega) using gene-specific downstream primers targeting the HCV p22 core region, with minor

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Table 1. Detection of hepatitis C virus (HCV) RNA and blood in biological specimens obtained from 38 patients with HCV-positive serum specimens.

Assay	No. (%) of persons (n = 38)	95% CI
Blood detection with OBTI		
Nasal secretions	28 (73.7)	57.8–85.2
Sniffing straws	3 (7.9)	2.0–21.5
HCV RNA detection with PCR		
Nasal secretions	5 (13.2)	5.3–27.8
Sniffing straws	2 (5.3)	0.5–18.2

modification of the upstream primer (410R-5′-ATGTACCCCA-TGAGGTCGGC-3′). HCV cDNA was amplified by PCR with 40 cycles of denaturation (94°C for 30 s), annealing (58°C for 30 s), and elongation (72°C for 45 s) with primers 406F-5′-TAGACCGTGCACCATGAGC-3′ and 410R. PCR products were detected by Southern blot using ^{32}P-labeled probe (5′-AGGAAGACTTCCGAGCGGTCGCAA-3′).

HCV cDNA was amplified from randomly selected HCV-positive blood samples with use of high-fidelity Pfu polymerase (Perkin Elmer) using 410R and 406F primers and cloned into a TA cloning vector (Invitrogen). The pTA_HCV was used to prepare standard curves ranging from 1×10^6 to 10 copies of HCV mRNA, which were run in parallel to each set of samples. The intensity of DNA bands was evaluated by densitometry using the Kodak Image Analysis System; the HCV load for the test sample was calculated on the basis of the numeric value derived from the HCV titration curve. HCV load was calculated as the number of copies per milliliter for blood specimens and as the number of copies per sample for nasal secretions and implements.

Traces of blood in nasal secretions and sniffing implements were detected by Hexagon OBTI Kit (BLUESTAR Forensic). Titration curves were prepared using human hemoglobin (Sigma) in 2-fold dilutions ranging from 10 to 0.1 μg/mL. The concentration of blood in each sample was established by comparing the OBTI intensity between the sample and the hemoglobin titration curve.

Nasal cavity pathology was assessed for each patient by anterior nasal examination, rendering diagnoses on 8 nasal pathologies. Rhinitis was diagnosed on the basis of the classic symptoms of mucosal and nasal secretion appearance [9]. Rhinosinusitis was defined by symptomatic inflammation of the paranasal sinuses and nasal cavity [10].

Sample prevalences of HCV RNA and occult blood in nasal secretions and on sniffing implements were estimated. Ninety-five percent CIs were calculated around point estimates using the adjusted Wald method. Descriptive statistics were calculated for sample descriptors and measures of nasal pathology. Our limited sample size precluded statistical tests of significance (e.g., associations between virological and clinical variables).

Results. All 38 patients had chronic, active hepatitis C. The serum HCV load ranged from 250 to 5,000,000 copies/mL (median, 5000 copies/mL). Recent liver biopsies had been performed for 6 patients; all indicated chronic liver disease, with stages ranging from 1 to 4. Recent alanine aminotransferase levels were available for 17 patients; the mean level (± SD) was 46.7 ± 26.7 U/L (range, 16–118 U/L). Antibody screening revealed that 34% of subjects were positive for antibodies to HIV, and 45% were positive for antibodies to hepatitis B virus.

Trace amounts of blood were detected in 28 (74%) of 38 nasal secretion samples (range, 0.1–10 μg/mL) and on 3 (8%) of the 38 sniffing implements (range, 0.1–2 μg/mL). HCV RNA was detected in 5 nasal secretion samples (13%; HCV RNA level range, 10–100 copies/sample) and on 2 sniffing implements (5%; HCV RNA level, 50 and 100,000 copies/sample). Prevalence estimates suggest a wide discrepancy between the presence of blood (74%) and the presence of HCV RNA (13%) in the nasal secretion samples (table 1). Of the 5 HCV RNA–positive nasal secretion samples, only 3 had traces of occult blood; of the 28 samples containing occult blood, 25 were negative for HCV RNA (figure 1).

The prevalence of rhinitis in this cohort was high (71%) (table 2). In contrast, the prevalence of rhinosinusitis (11%) is consistent with that of the general population. More than 40% of subjects experienced rhinorrhea or nasal congestion at least once per week, 8% reported nose bleeds at least once per week, and 8% and 16% reported mucosal lesions and crusting, respectively. Approximately one-half of the subjects attributed these symptoms to intranasal drug use. Four persons (11%) were observed to have nasal septal perforations; 1 (3%) had a nasopalatal perforation; and 6 (16%) displayed symptoms of saddlenose deformation. These pathologies have been associated with advanced nasal cavity deterioration associated with chronic intranasal drug use [11].

Discussion. Our findings revealed a high prevalence of blood (74%) in the nasal secretions of HCV-positive long-term drug sniffers. We also confirmed that HCV RNA was present in the nasal secretions of a substantial proportion (13%) of this cohort. Most significantly, this study demonstrated that both blood and HCV particles can be transferred onto sniffing im-

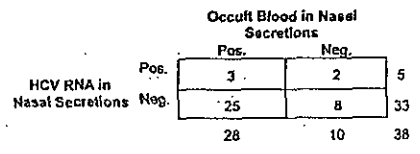

Figure 1. Hepatitis C virus (HCV) RNA and occult blood in nasal secretions.

Table 2. Frequency of nasal pathology symptoms among intranasal drug users.

Symptom	No. (%) of subjects (n = 38)
Findings of an anterior nasal clinical examination	
Loss of nasal hairs	4 (10.5)
Rhinitis	27 (71.1)
Rhinosinusitis	4 (10.5)
Presence of nasal crusting and/or scabbing	6 (15.8)
Sores or erosion of nasal mucosa	3 (7.9)
Saddlenose deformation	6 (15.8)
Nasopalatal perforation	1 (2.6)
Nasal septum perforation	4 (10.5)
Self-reported nasal pathology	
Frequency of nosebleeds in the past year	
Never or rarely	26 (68.4)
Once or a few times per month	9 (23.7)
Once or a few times per week	2 (5.3)
Once or more per day	1 (2.6)
Experienced a runny or stuffy nose in the past year	
Never or rarely	16 (42.1)
Once or a few times per month	6 (15.8)
Once or a few times per week	13 (34.2)
Once or more per day	3 (7.9)
Reason for nasal symptoms	
Allergies	19 (50.0)
Cold or influenza	10 (26.3)
Drug sniffing	21 (55.3)
"Have you ever noticed any of the following problems with your nose due to drug sniffing?"	
Scabs in the nose	14 (36.8)
Sores in the nose	8 (21.1)
Poor sense of smell	13 (34.2)
Sinus pain	13 (34.2)
Headaches located in the forehead	16 (42.1)
Double vision	5 (13.2)
"Has a doctor or other health care professional ever told you that the inside of your nose is damaged in any way from sniffing drugs?"	7 (18.4)

plements (i.e., straws) during simulated intranasal drug use. Studies have shown that HCV can remain viable on environmental surfaces for up to 16 h, but little is know about the quantity of virus required for transmission [12]. The prevalences of HCV in the nasal secretions and on sniffing straws are likely conservative estimates. It is reasonable to assume that HCV will be present in the nasal secretions with greater frequency and quantity during episodes of active drug sniffing, which may exacerbate discharge of nasal fluids and blood.

Data in table 1 contradict the assumption that, in persons with HCV-positive serum specimens, detection of blood implies the presence of HCV. This discrepancy may be explained by 2 factors. First, the 2 assays (PCR and OBTI) were not performed on the same samples. Second, the OBTI assay for blood detects immune complexes between human hemoglobin (hHb) and monoclonal anti-hHb antibodies, which can occur even in the absence of viable cells. In contrast, PCR can only detect HCV RNA from intact particles. Therefore, the discrepancy between the high prevalence of occult blood and relatively low detection of HCV RNA in nasal secretions may be associated with the rapid deterioration of viral RNA in the nasal environment or the destruction of viral particles by mucosal immunity. If the viability of HCV particles in nasal secretions is moderated by nasal pathology or immunity, this might help explain conflicting epidemiological findings in which these moderating factors are not considered.

This study establishes the validity of 2 primary virological preconditions necessary for intranasal HCV transmission: (1)

the presence of blood and HCV in the nasal secretions of intranasal drug users, and (2) the transference of blood and HCV from the nasal cavity onto sniffing implements, which are often shared by intranasal drug users. Moreover, the frequency and severity of nasal pathologies observed in this cohort might aggravate conditions that facilitate intranasal HCV transmission. Consequently, these findings lend important virological and clinical support to the intranasal HCV transmission hypothesis. In addition, detection of HCV in nasal secretions advances the debate regarding potential iatrogenic and nosocomial transmission of HCV in the context of ear, nose, and throat and related clinical practices. More research is needed to confirm intranasal transmission as a mode of viral infection and to determine its impact on the wider epidemic of HCV infection.

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別紙様式第２－１

医薬品　研究報告 調査報告書

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研究報告の概要	使用上の注意記載状況・その他参考事項等
E 型肝炎ウイルス (HEV) はヘペウイルス属に分類され、エンベロープがなく、直径約 27～34nm のウイルスで、糞便から経口、食物媒介および血液媒介経路で伝播され、ヒトの肝炎の原因となる。 今までの報告では、56℃30 分間の加熱で 4 つの HEV 株が不活化され、別の報告では糞便由来の 3 つの HEV 分離株は、56℃または 60℃60 分の加熱で不活化されたが、熱抵抗性の特性は株間でわずかに異なったとされている。 本研究では、日本で発見された遺伝子型 3 と 4 の 4 つの HEV 分離株を用いて、アルブミン及びフィブリノゲンにおける液状加熱、乾燥加熱およびウイルス除去膜ろ過による HEV 不活化/除去能を検討した。 その結果、25％アルブミン (液状加熱段階直前に採取) では、60℃で 5 時間の加熱を行ったが、いずれの HEV 分離株も熱抵抗性を示し、5 時間加熱後も感染力が検出され、LRF (log reduction factor) はそれぞれ 2.0、2.0、1.0 および 2.2 以上であった。 フィブリノゲン (2.0 w/v％塩酸 L-アルギニン含有、乾燥加熱段階直前に採取) では、60℃で 72 時間処理したところ、感染力が検出された。 また、ウイルス除去膜では、いずれの HEV 分離株も、孔径 19nm および 15nm では検出限界以下まで除去されたが、35nm では大量の HEV が検出され、HEV の粒子サイズは既に電子顕微鏡分析で報告されているように約 35nm であることが示唆された。	

報告企業の意見	今後の対応
HEV の不活化、除去に関する情報である。 現在まで、血漿分画製剤による伝播の報告はなく、製造工程中には複数のウイルス不活化除去工程を設けているが、今後とも関連情報の収集に努める。	今後とも HEV に関する情報に留意し、関連情報の収集に留意していく。

(5)

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Extent of hepatitis E virus elimination is affected by stabilizers present in plasma products and pore size of nanofilters

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Vox Sanguinis

Background and Objective To investigate the physico-chemical properties of hepatitis E virus (HEV) with regard to inactivation/removal, we have studied four isolates with respect to sensitivity to heat during liquid/dry-heating as well as removal by nanofiltration.

Materials and Methods Hepatitis E virus in an albumin solution or phosphate-buffered saline (PBS) was liquid-heated at 60°C for a preset time. HEV in a freeze-dried fibrinogen containing stabilizers was also dry-heated at 60 or 80°C for a preset time. In addition, to clarify the removal of HEV, the purified virus in PBS was filtered using several types of virus-removal filter (nanofilters) that have different pore sizes. HEV infectivity or genome equivalents before and after the treatments were assayed by a semiquantitative cell-based infectivity assay or quantitative polymerase chain reaction assay, respectively.

Results Hepatitis E virus isolates in albumin solutions were inactivated slowly at 60°C for 5 h and the resultant log reduction factor (LRF) was from 1·0 to ≥ 2·2, whereas the virus in PBS was inactivated quickly to below the detection limit and the LRF was ≥ 2·4 to ≥ 3·7. The virus in a freeze dried fibrinogen containing trisodium citrate dihydrate and L-arginine hydrochloride as stabilizers was inactivated slowly and the LRF was 2·0 and 3·0, respectively, of the 72 h at 60°C, but inactivated to below the detection limit within 24 h at 80°C with an LRF of ≥ 4·0. The virus in PBS was also confirmed as to be approximately 35 nm in diameter by nanofiltration. These results are useful for evaluating viral safety against HEV contamination in blood products.

Conclusion The sensitivity of HEV to heat was shown to vary greatly depending on the heating conditions. On the other hand, the HEV particles were completely removed using 20-nm nanofilters. However, each inactivation/removal step should be carefully evaluated with respect to the HEV inactivation/removal capacity, which may be influenced by processing conditions such as the stabilizers used for blood products.

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Key words: dry-heating, heat inactivation, HEV, liquid-heating, nanofiltration.

Introduction

Hepatitis E virus (HEV), classified in the genus *Hepevirus*, is a causative agent of human hepatitis. The virus capsid is non-enveloped and the nucleocapsid containing positive-sense single-stranded RNA has a diameter of 27–34 nm [1]. HEV

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is also endemic in humans, swine and several wild animals such as deers and boars, suggesting that hepatitis E is a zoonosis [2,3].

The virus has been shown to be transmitted by faecal-oral, food-borne and blood-borne routes [1,4–7]. Four genotypes of HEV that infect humans have been identified, three of which, genotypes 1, 3 and 4, have also been isolated from swine and commercial swine liver [1,8,9]. Zoonotic food-borne transmission of HEV was shown to be one reason for the occurrence of a severe form of hepatitis E in Hokkaido, Japan, and HEV genotype and the presence of an underlying disease influenced the severity of the hepatitis E infection [10]. In addition, the prevalence of HEV RNA or anti HEV immunoglobulin G (IgG)-positive blood donors in Hokkaido was 0·01% (56/432,167) and 3·9%, respectively [11]. These reports also suggested that a small but significant proportion of blood donors in Japan with or without elevated alanine aminotransferase (ALT) levels are viremic and are potentially able to cause transfusion-associated hepatitis E. Note that anti-HEV IgG and HEV levels in pooled plasma have not been reported yet. Thus, these data may indicate the need for precautions against the potential risk of transfusion-transmitted HEV infection, as previously discussed [12]. In addition to foods, the safety of plasma-derived products with respect to HEV may be an important issue and each product should be evaluated for safety against HEV contamination.

Huang *et al.* reported that four HEV strains in culture media containing 2% calf serum were inactivated and that residual infectivity was not detected after heating at 56°C for 30 min [13]. Emerson *et al.* reported that three HEV isolates derived from faeces including genotypes 1 and 2 were inactivated after 60 min at 56 or 60°C, but the heat-resistance properties differed slightly between the strains used. A strain that was slightly more resistant to heating showed some residual infectivity (< 1%) after 1 h at 56°C [14]. Tanaka *et al.* also reported that an HEV isolate in a faecal suspension in Tris-HCl buffer was inactivated and that residual infectivity was not detected after heating at 70°C for 10 min, whereas residual infectivity was detected after 30 min at 56°C [15]. Unfortunately, these studies did not evaluate the log reduction of infectivity and kinetic pattern of inactivation.

There have been no reports of HEV transmission via plasma-derived products that contain various kinds of proteins at high concentrations and also various types of stabilizers. However, investigative methods with log reduction and/or general information on HEV regarding the contamination of blood products have been required. In this study, we investigated the impact on the ability to inactivate HEV during liquid/dry-heating and viral particle removal by nanofiltration in plasma protein preparations using four HEV isolates found in Japan and belonging to genotypes 3 and 4.

Materials and methods

Viral isolates

Isolates from four different HEV clusters were used, that is, genotype 3_{SP} [swJB-E, cluster SP (3e), GENBANK (in preparation by Yamate *et al.*)], genotype 3_{US} [swJB-M, cluster US (3a), GENBANK (in preparation by Yamate *et al.*)], genotype $3_{JP\alpha}$ [swJB-N, unclassified cluster, GENBANK (in preparation by Tsunemistu *et al.*)], and genotype 4_{JP} [swJB-H, cluster JP (4c), GENBANK (in preparation by Yamate *et al.*)] (Table 1). These viruses were derived from faeces of infected swine in Japan. The origins of swJB-H, swJB-E and swJB-M were naturally infected swine faeces, while swJB-N was from faeces of experimentally infected swine (Highland strain, kindly provided by Dr Hiroshi Tsunemitsu, National Institute of Animal Health, Japan).

Table 1 Details of viral isolates used

Genotype[a]	Isolation ID	Viral titre: HEV genome[b]	Viral titre: HEV infectivity[c]	Used for
$3_{JP\alpha}$	swJB-N2	6·3	3·8	Liquid-heating, nanofiltration
3_{US}	swJB-M5	7·2	4·8	Nanofiltration, dry-heating
	swJB-M8	8·4	5·3[d]	Liquid-heating
3_{SP}	swJB-E8	7·5	4·8	Dry-heating
	swJB-E10	7·7	5·8[e]	Liquid-heating, nano-filtration
4_{JP}	swJB-H1	7·0	–	Nanofiltration
	swJB-H1/H7	7·0/7·4	4·8[f]	Liquid-heating
	swJB-H7	7·4	3·2[d]	Liquid-heating
	swJB-H8	6·8	3·8	Liquid-heating
	swJB-H21[g]	7·2	3·8	Liquid-heating

[a]The genotypes and clusters of isolates were grouped as described by Takahashi *et al.* and Lu *et al.* [24,25].

[b]Genome amount is indicated by log copies per ml. For swJB-M, specific primer sets and probes '(sense primer F2: 5′-TCGTGTACAAACCGAGATTC-3′, anti-sense primer R2: 5′-GCCCGGCAATATTGTTCTA-3′, Probe Flu2: 5′-GATGCAACCCCGGCAGTTGGTTTTC-FITC-3′ and Probe LC2: 5′-LCRed640-GCCCTGAGGTACTCTGGAATCATCCTATCC-3′)' were designed and used. For the other isolates, the primer set and probe (HE86, HE87 and FAMlabeled probe FHE88) designed by Jothikumar *et al.* [26] were used.

[c]Infectivity titre is given as log dilution non-detectable end-point per ml.

[d]Mean titre of two ([e]three) independent experiments.

[f]Mixture of H1 and H7 used.

[g]This isolate is derived from faeces of an experimentally infected piglet.

Isolation and purification of virus

Faecal samples (10 g) were resuspended with 100 ml of phosphate-buffered saline (PBS) and centrifuged at 1600 *g* for 10 min and the supernatant retained. Pellets were resuspended in 50 ml of PBS and the suspension was centrifuged again under the same conditions. Resultant pellets were resuspended with 25 ml of PBS and the suspension was centrifuged again. All these three supernatants were pooled and were filtered using an AP filter (AP2504700, Millipore, Billerica, MA, USA). After centrifugation at 10 000 *g* for 30 min, the supernatant was filtered through four sequential filters (5·0 μm; SMWP04700, 1·2 μm; RAWP04700, 0·8 μm; AAWP04700, and finally 0·45 μm; HAWP04700, Millipore). Then polyethylene glycol (PEG) 6000 (Wako Pure Chemical Industries, Osaka, Japan) and sodium chloride up to final concentrations of 8% (w/v) and 2·4% (w/v), respectively, were added to the final filtrate. The solution was stirred for 10 min and incubated overnight at 4°C. The solution was centrifuged at 10 000 *g* for 30 min and the precipitate was resuspended with one-tenth the volume of the original solution of PBS prior to the addition of PEG. The solution was sonicated and centrifuged at 4000 *g* for 15 min at 4°C. The resultant supernatant was filtered in two steps (0·45 μm; SLHV033RS and 0·22 μm; SLGV033RS, Millipore), and the filtrate was aliquoted and stored at −80°C as HEV stock. Isolated HEV samples were allocated an isolation ID and preparation lot number.

Hepatitis E virus stocks were further purified for filtration experiments. The viral stocks in PBS were treated with 1% (v/v) Tween-80 (Wako Pure Chemical Industries) and 0·3% (v/v) Tri-n-butyl Phosphate (TNBP, Sigma, St. Louis, MO, USA) for 1 h at 30°C and then the solutions were ultracentrifuged at 150 000 *g* for 3 h at 4°C. The precipitates were resuspended in PBS and subsequently sonicated and centrifuged at 4000 *g* for 15 min at 4°C. The supernatants were filtered by sequential 0·22 and 0·1 μm filtration [SLGV033RS (0·22 μm) and SLVV033RS (0·1 μm); Millipore] and the filtrate was aliquoted and stored at −80°C as purified HEV stock. In addition, HEV Genotype 3_{SP} derived from the culture media of infected A549 cells was treated with detergent alone, as described above, and subsequently used for filtration experiments.

Quantitative HEV RNA assay for each isolate

The total HEV RNA in each sample was extracted using the RNeasy Mini Kit (cat. 74104; Qiagen GmbH, Hilden, Germany) and then quantified by polymerase chain reaction (PCR) using specific primers. The copy number of swJB-M was quantified using specified primers and probes set from the light cycler (LC) RNA Amplification Kit Hybridization Probes (Roche Diagnostics, Basel, Switzerland) and LC quick system 350S (Roche Diagnostic). The assay conditions were as follows: reagents; 4·0 μl of 5× LC reverse transcription (RT)-PCR Mix HybProbe (Roche Diagnostic), 3·2 μl of 25 mM $MgCl_2$, 2·0 μl of 5 pmol/μl primer F+R, 2·0 μl of 2 pmol/μl probe Flu+LC, 3·4 μl of water, 0·4 μl of LC RT-PCR enzyme mix and 5·0 μl of template (total 20 μl), and reaction; 55°C 10 min, 95°C 30 second, 45 cycles of 95°C 5 second, 60°C 15 second, 72°C 13 second and subsequently 40°C 30 second. The copy number of ORF3 for swJB-N, swJB-E and swJB-H (genotypes $3_{JPα}$, 3_{SP} and 4_{JP}) was also quantified using a QuantiTect Probe RT-PCR Kit (Qiagen) and Applied Biosystems 7500 (Applied Biosystems, Foster City, CA, USA). The assay conditions were as follows: reagents; 25 μl of 2× QuantiTect Probe RT-PCR Master Mix (Qiagen GmbH), 1·0 μl of 20 μM primer Mix, 0·5 μl of 10 μM Probe, 0·5 μl of QuantiTect RT Mix, 13·0 μl of water and 10 μl of template (total 50 μl), and reaction; 50°C 30 min, 95°C 15 min, 45 cycles of 95°C 15 second and 60°C 35 second.

Infectivity assay for HEV

Infectivity of HEV was assayed according to Huang *et al.* [13] with minor modifications. A549 cells (kindly provided by Dr Takaaki Nakaya, Research Institute for Microbial Diseases, Osaka University) were cultured in DMEM (cat. 11995-065, Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (cat. SH30071·03; Hyclone, Logan, UT, USA), 100 U/ml penicillin, 100 μg/ml streptomycin (cat. 15140-122, Invitrogen) and Insulin-Transferrin-Selenium-X (ITS-X) supplement (cat. 51500-056, Invitrogen) at 37°C in 5% CO_2 in air. The composition of the medium used for the viral assay was Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, ITS-X supplement and 30 mM $MgCl_2$ (cat. 135-00165, Wako Pure Chemical Industries) at 37°C in 5% CO_2 in air. For the infectivity assay, A549 cells were seeded in a 12-well microplate ($3·6 \times 10^5$ cells/ml, 2 ml/well). After an overnight culture, the cells were inoculated with serial 10-fold dilutions of the virus stock solution (0·3 ml/well). On day 7 of culture, HEV RNA in cultured cells was assayed using the HEV RNA assay method described above. The infectivity of each stock of isolate used was determined from the dilution end-point where no RNA was detected.

Heat sensitivity of HEV during liquid- and dry-heating

Hepatitis E virus isolates were ultracentrifuged at 150 000 *g* for 3 h at 4°C. The resultant pellets were resuspended with PBS or a 25% albumin solution that was collected just before the heating step in the manufacture of Kenketsu Albumin-Wf (Benesis, Osaka, Japan) as a stabilizer. These samples were aliquoted at 0·5 ml per tube and incubated in a water bath at 60°C for preset times (0, 0·5, 1, 2 and 5 h). After quickly

cooling, the residual infectivity of the sample was determined as described above.

The HEV precipitates described above were also resuspended with a Fibrinogen solution containing 1·3% (w/v) trisodium citrate dihydrate and 2·0% (w/v) L-arginine hydrochloride as a stabilizer that was collected just before the dry-heating step in the manufacture of Fibrinogen HT-Wf (Benesis). The HEV solutions were aliquoted at 2·0 ml/vial and freeze-dried using an optimized freeze drying cycle (programme) for this product (freeze dry systems cat. 7948020 and 7934024, Labconco, Kansas City, MO, USA). The freeze-dried samples in the vials were closed under vacuum. The vials were then heated at 60 or 80°C in a drying oven (cat. DK43; Yamato Scientific, Tokyo, Japan) for 72 h. The heated samples were cooled quickly and stored at 4°C until the assaying. Residual infectivity was assayed as described above. In addition, the residual water content of mock-infected samples prepared using the same freeze drier programme and conditions without spiking with HEV were assayed using the loss on drying test method described previously [16].

Removal of HEV by nanofiltration

Hepatitis E virus stocks that were detergent-treated, as described above, were thawed, concentrated, if required, sonicated and filtered using 0·22 μm (0·22 μm; SLGV033RS, Millipore) and Bemberg Microporous Membrane (BMM) filter (Planova®-75N (72 ± 4 nm, 0·001 m^2); Asahi Kasei Medical, Tokyo, Japan) immediately prior to nanofiltration. The viral samples were subjected to nanofiltration using BMM-35N (35 ± 2 nm), -20N (19 ± 2 nm) and -15N (15 ± 2 nm; Asahi Kasei Medical) under conditions where 2-ml samples were applied to 10^{-5} m^2 filters with 50 kPa and dead end filtration. The quantities of HEV RNA before and after filtration were measured using the quantitative HEV RNA assay described above.

Results

Viral preparations

Isolates from four different clusters including two genotypes were prepared and each isolate was evaluated regarding genome and infectious titre in the stocks.

We evaluated the appropriateness of the method to determine the HEV infectious titre by semiquantitative PCR (data not shown). The levels of HEV RNA in the infected cells were higher at 3 and 7 days post-infection (dpi) than at 0 dpi. The titres obtained were not consistent on 3 dpi whereas the results were consistent on 7 dpi. Therefore, we decided that the titre of HEV should be determined on 7 dpi. According to our data, about 1000 copies of the genome per infectious unit were observed in our system. The infectious titres in the HEV stocks of the viruses are summarized in Table 1.

Heat sensitivity of HEV

The heat-inactivation kinetics of HEV isolates from four clusters including two genotypes during liquid-heating using 25% albumin and PBS at 60°C for 5 h was evaluated. All isolates in PBS were inactivated below the detectable infectivity limit within 30 min at 60°C and showed a rapid inactivation. The log reduction factor (LRF) of genotype $3_{JPα}$, 3_{SP}, 3_{US} and 4_{JP} was ≥ 2·7, ≥ 3·7, ≥ 3·7 and ≥ 2·4, respectively. In contrast, all HEV isolates in the 25% albumin solution showed heat resistance, and residual infectivity was detected even in the samples heated for 5 h and the LRF was 2·0, 2·0, 1·0 and ≥ 2·2, respectively (Fig. 1).

The heat-inactivation kinetics of Genotype 3_{US} and 3_{SP} in fibrinogen during dry-heating was also evaluated. The water content of freeze-dried samples containing the two HEVs was < 0·3%. Residual infectivity was not detected with the LRF

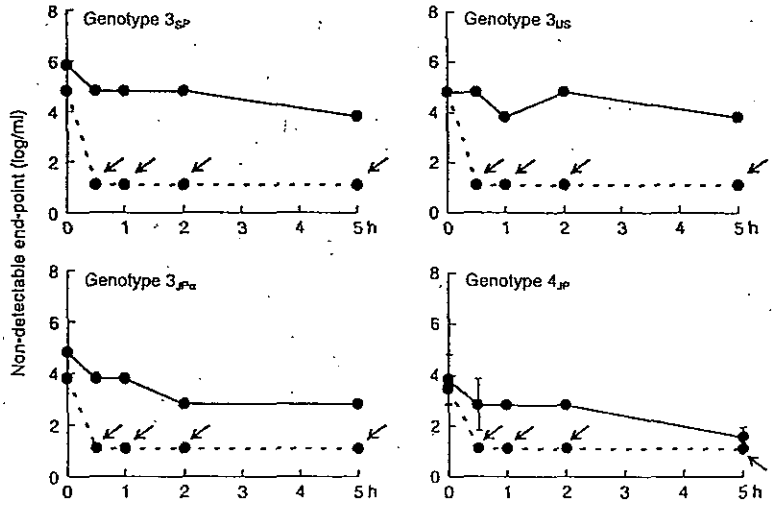

Fig. 1 Inactivation kinetics of the four HEV isolates during liquid-heating. Solid lines: HEV in 25% albumin. Broken lines: HEV in PBS. Arrow: infectious virus was not detected. Genotype 4_{JP}: $n = 3$.

