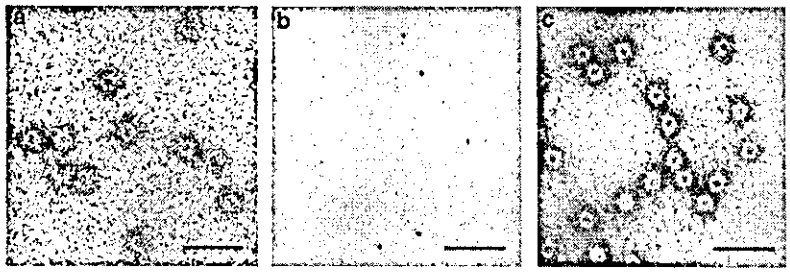


*Figure 1 Electron micrographs of HEV-VLPs: (a) purified HEV-VLPs before treatment; (b) disassembled HEV-VLPs after treatment of VLPs with EGTA and DTT; and (c) refolded HEV-VLPs in the presence of $CaCl_2$, DMSO and DNA. Bars represent 50 nm.*

human acute hepatitis by fecal–oral transmission. HEV first infects epithelial cells of the small intestine and then reaches the liver through the portal vein. It has recently been reported that overexpression of a part of open reading frame 2 (ORF2) in a baculovirus expression system results in the assembly of this protein into a VLP.[10] We have also reported that VLPs carrying foreign epitopes elicit strong mucosal and systemic immune responses to both the VLPs and exogenous epitopes without the requirement of any kind of adjuvant when orally administered to mice.[11]

Since infection with human immunodeficiency virus (HIV) most likely occurs through exposure of mucosal tissue to the virus, HIV-specific immune responses at mucosal sites are critical for the initial control of infection. Therefore, a nonreplicating vaccine vector that elicits mucosal immunity by oral administration would be a powerful HIV vaccine. In the present study, we found that unrelated plasmid constructs can be encapsulated into HEV-VLPs and delivered to the intestinal mucosa by oral administration. HIV DNA vaccine-loaded HEV-VLPs can elicit mucosal and systemic cellular as well as humoral immune responses by oral administration.

## Results

### *In vitro refolding of VLPs*

The HEV-VLPs produced by a recombinant baculovirus system were disassembled by the removal of calcium ions (Figure 1b). When calcium ions were supplemented to the disrupted VLPs in the presence of plasmid DNA, the DNA was encapsulated into the refolded VLPs (Figure 1c). No significant morphological difference due to the VLP disassembling–refolding process was observed under an electron microscope.

### *Density shifts of VLPs and amount of plasmid DNA after DNA encapsulation*

Plasmid DNA encapsulation in the refolded VLPs was confirmed by CsCl equilibrium gradient centrifugation. VLP density is greater when loaded with a DNA plasmid. A heavier density gradient peak was present only when DNA was incorporated into the VLPs (Figure 2d). A single lighter density peak was produced for VLPs alone (Figure 2a), refolded VLPs (Figure 2b) and intact VLPs in the presence of plasmid DNA (Figure 2c). Despite the various sizes of plasmid DNA used for

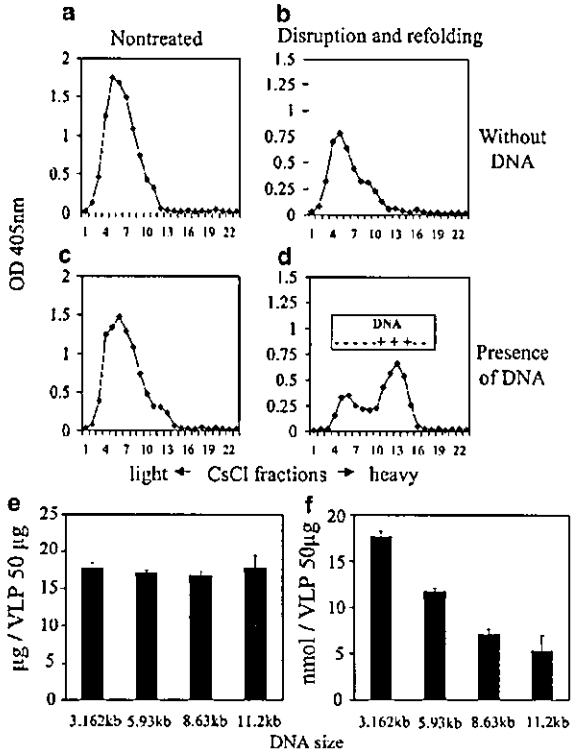


*Figure 2 CsCl gradient profiles of intact and refolded VLPs. No DNA added: (a) intact; (b) refolded. DNA added: (c) intact; (d) refolded. The amount of DNA encapsulated in VLPs is expressed as μg (e) and molality (f) per 50 μg VLP protein.*

encapsulation, the amounts of plasmid in VLPs were almost the same (17–19 μg per 50 μg of HEV-VLPs) (Figure 2e and f). A solution with a high concentration of plasmid DNA showed high viscosity, and VLPs including DNA were not obtained for general use in experiments. Based on these results, we used this amount (1 mg/ml) as the optimal concentration (data not shown).

### *Gene transfer by HEV-VLPs*

Initially, four cell lines derived from mice, rabbits, monkeys and humans were studied for their ability to

transfer genes *in vitro*. The fluorescence of GFP-expressing cells was observed under a fluorescence microscope. Although the percentages of fluorescence-positive cells were not so high (11.2% of NIH3T3 cells, 19.6% of RK-13 cells, 21.0% of COS-7 cells and 20.1% of HepG2 cells), all of the cell lines used in this study showed positive reactions (Figure 3). In contrast, no fluorescence-positive cells were observed when the cells were incubated with plasmid DNA alone or intact VLPs in the presence of plasmid DNA (data not shown). We next tried gene transduction *in vivo*. Mice that had orally received a vaccine of DNA expressing HIV env gp120 of the NL432 strain (pJWNL432) that was encapsulated in VLPs were killed 2 days after immunization, and the expression of HIV env protein in the digestive tract was examined. HIV env protein was found in epithelial cells of the small intestine by immunohistochemistry (Figure 4), indicating that the HEV structure necessary for the entry of HEV into target cells had been preserved in refolded VLPs and that the DNA encapsulated in HEV-VLPs had been delivered to intestinal tissues.

### *Systemic and mucosal HIV-specific humoral immune responses in mice that had orally received a vaccine of HIV DNA encapsulated in VLPs*

Mice were orally or subcutaneously immunized four times at 1-week intervals with pJWNL432 either naked or encapsulated in HEV-VLPs. The serum levels of HIV env-specific IgG antibodies in mice that had received loaded VLPs were significantly higher than those in mice that had received naked DNA ($P<0.05$ at 12 wpi, Figure 5a and e). Moreover, specific IgA was detected at high levels in sera of mice that had received loaded VLPs but not in sera of mice that had been immunized subcutaneously ($P<0.05$ at 12 wpi, Figure 5b and f). HIV env-specific IgA was only detected in fecal extracts of mice that had orally received pJWNL432-encapsulated HEV-VLPs (Figure 5d and h). No specific IgG was detected in any of the fecal samples (Figure 5c and g). The levels of HIV env-specific IgG antibodies detected in sera from subcutaneously and orally immunized mice were the same (Figure 5a and e). HEV-specific IgA was detected in both sera and fecal extracts of mice that had been orally administered VLP but not in sera or fecal extracts of mice that had been immunized subcutaneously (Figure 5j and l). Both orally and subcutaneously immunized mice showed HEV-specific IgG in sera (Figure 5i) and fecal extracts (Figure 5k).

### *Elicitation of HIV-specific cytotoxic T lymphocytes at systemic and mucosal sites by oral administration of a vaccination of HIV DNA encapsulated in VLPs*

Cytotoxic T lymphocyte (CTL) responses in the spleen, mesenteric lymph nodes (MLN) and Payer's patches (PP) were investigated at 5 weeks after the first immunization. Mice that had orally received pJWNL432 encapsulated in HEV-VLPs showed HIV env epitope-specific CTL responses in the spleen, MLN and PP, whereas cells from the same tissues in mice that had received naked DNA vaccine did not show any CTL activity (Figure 6a). The P18 peptide is a dominant HIV env CTL and Th cell epitope in BALB/c mice and is restricted to the H-2D$^d$ allele. These effector cell functions derived from our experiments were inhibited by either anti-CD8 or -H-2D$^d$ monoclonal antibody (mAb) (Figure 6b,c), indicating that oral immunization of mice with a vaccine of HIV env DNA-encapsulated HEV-VLPs elicited CD8$^+$ and MHC class I-restricted CTLs both locally and systemically.

## *Discussion*

A large number of pathogens gain access to the human body via mucosa such as oral, nasal or genital mucosa. The best defense against these predominantly mucosal

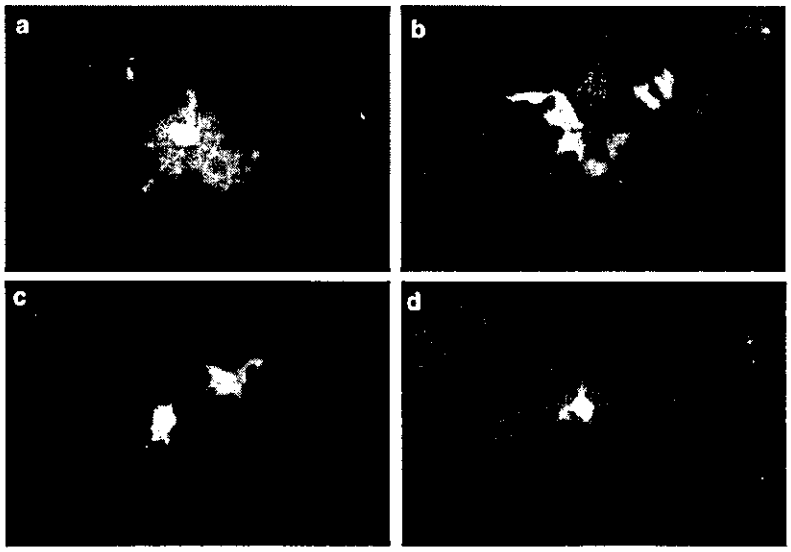


*Figure 3 Expression of GFP in cells transfected with plasmid DNA encapsulated in HEV-VLPs: (a) NIH/3T3 cells (mouse); (b) RK-13 cells (rabbit); (c) COS-7 cells (monkey); and (d) HepG2 cells (human).*

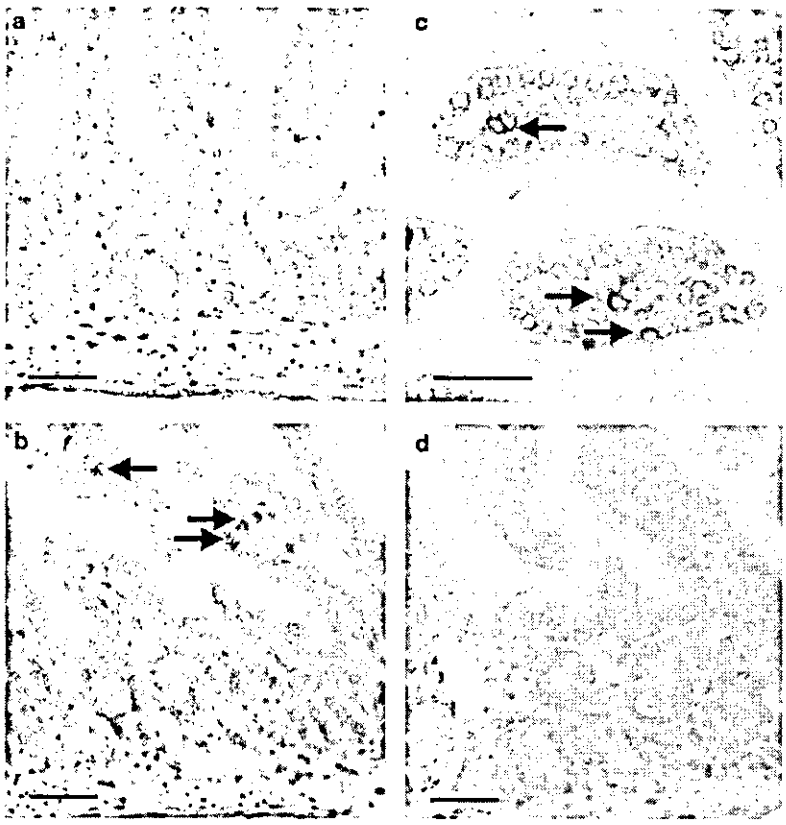


*Figure 4 Immunostaining of serial sections of small intestine tissue from mice 2 days after oral administration of pJWNL432-encapsulated VLPs. HIV env proteins were observed in epithelial cells (arrow) (b, c), and control mAb did not show any positive reactions (d). Control mice were also administered pJWNL432 without VLP encapsulation (a). Bar marker represents 50 μm.*

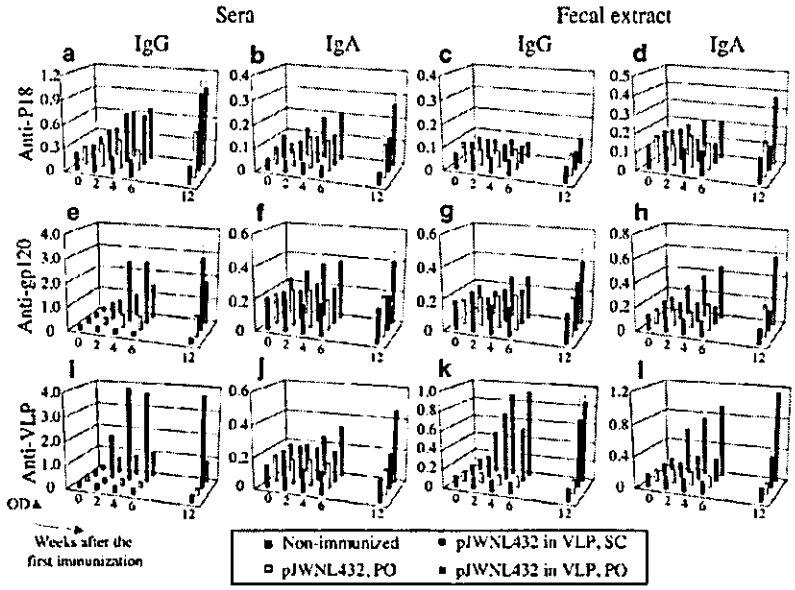


*Figure 5 IgG (a, c, e ,g, i and k) and IgA (b, d, f, h, j and l) levels in sera (a, b, e, f, i and j) and fecal extracts (c, d, g, h, k and l) of immunized mice. Mice were orally (■) or subcutaneously (□) administered pJWNL432 encapsulated in VLP or naked (■). Symbols indicate HIV env-specific antibody levels. Background levels to HIV env in nonimmunized mice (▩) are also shown. The IgG and IgA antibody levels are expressed as OD at dilutions of 1:100 and 1:2 for serum and fecal extracts, respectively. The mean OD values ±s.e.s were obtained from five mice/group.*

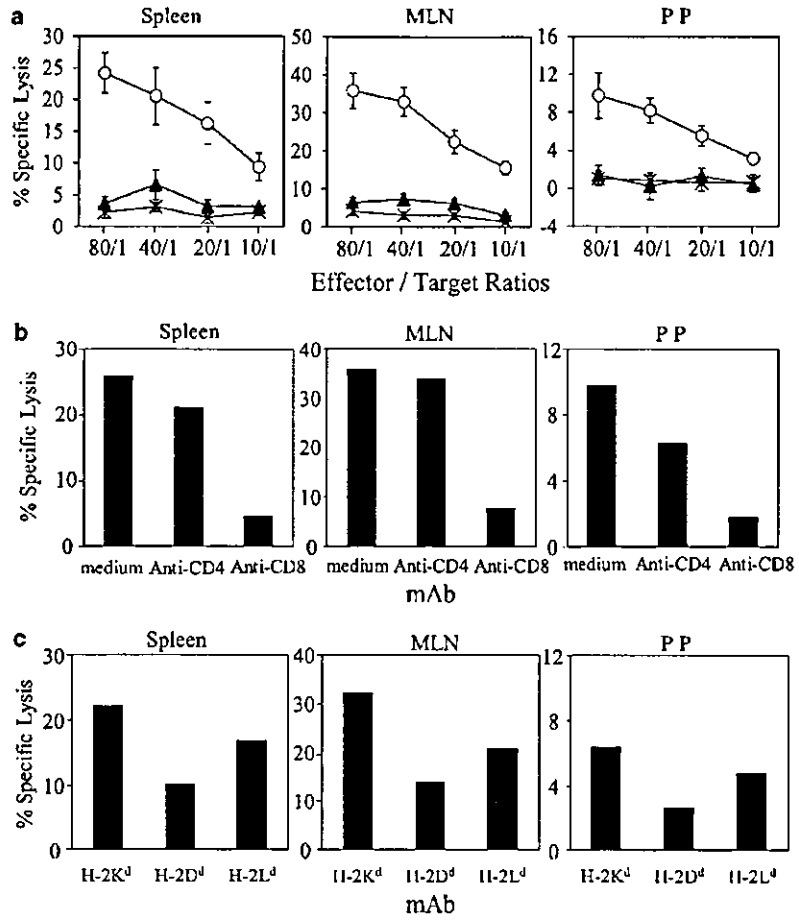


*Figure 6 Spleen, MLN and PP cells from mice orally administered pJWNL432-encapsulated VLPs elicited CTL. (a) Mice were orally administered pJWNL432 encapsulated in VLPs (circles) or naked (triangles). Results for nonimmunized controls are also shown ( × ). (b) Effector cells obtained from the spleen, MLN and PP cells of mice orally administered pJWNL432-encapsulated VLPs are mediated CD8$^+$ cells. Lytic activities of effector cells were assessed in the presence of anti CD4 mAb, anti-CD8 mAb or medium. Effector:target ratio was 80:1. (c) HIV env-specific lysis was restricted by MHC class I. Effector cells were examined for P18-specific lytic activities in the presence of anti-H-2K$^d$, anti-H-2D$^d$ or H-2L$^d$ mAb. The percentage of P18-specific lysis was calculated as (% lysis of target cells labeled with P18)−(% lysis of target cells labeled with control peptide). Each value is the mean percentage of the specific lysis values obtained from five mice.*

pathogens is mucosal vaccines that are capable of inducing both systemic and mucosal immunity. Recent evidence has shown that DNA vaccination can confer protection against a number of infectious agents, including viruses and bacteria, although peripheral immunization with naked DNA is less than optimal for stimulating mucosal immunity.[12,13] In fact, it is quite difficult to induce both mucosal and systemic immune responses by oral administration of naked DNA. This study demonstrated that an orally administered DNA vaccine encapsulated in an orally transmissible virus-derived VLP induced both mucosal and systemic immunity.

The delivery of a DNA vaccine for induction of mucosal immune responses is usually achieved by gene transfer to the upper nasopharynx-associated lymphoid tissue (NALT), upper airway, salivary glands and tonsils.[5,14] Despite its obvious convenience, oral administration is rarely successful, since it is quite difficult to protect plasmid DNA from the environment in the digestive tract. The efficacy of orally delivered DNA vaccine to NALT is improved by encapsulating plasmid DNA in poly (lactide-coglycolide) (PLG) microparticles for protection against the gastric environment.[15,16] The immune responses to particle-borne DNA immunizations by means such as utilization of a gene gun or PLG differ from those to DNA immunizations without particles.[13] It is thought that the microparticles are actively taken up by cells such as macrophages or M cells of PP of the small intestine and thus facilitate the presentation of antigens to local immune systems.[15,17] This mechanism is the same as that of gene gun immunization of a DNA vaccine, that is, phagocytic cells such as macrophages or dendritic cells take up plasmid DNA delivered by a gene gun. The delivered gene is expressed only in these cells.[18] Similarly, only mucosal immunity was induced in mice by oral administration of DNA-encapsulated PLG microparticles.[15,16] It is likely that the mechanism underlying immune recognition of

HEV-VLP infection is similar to that of direct intramuscular or subcutaneous DNA immunization without the use of particles. Protein expressed by HEV-VLP-infected cells is recognized by the immune surveillance system, resulting in the elicitation of Ag-specific immune responses. We showed in this study that genes could be expressed in epithelial cells in the small intestine after delivery by HEV-VLPs (Figure 4). It is plausible that HEV-VLPs, which are derived from an orally transmissible virus, were incorporated into HEV-permissive epithelial cells in the small intestine, because they retained structures and properties similar to those of HEV particles, producing an infection similar to that induced naturally.[19] The Ag-expressing cells might be recognized by intraepithelial lymphocytes or submucous antigen-presenting cells by the same mechanism as that in the case of general virus infection.

An HEV-VLP has several advantages as a vector of DNA. Firstly, in our experience, large amounts can be easily obtained from standard cultivation protocols compared with the amounts of other VLPs obtained. The yield of purified HEV-VLPs collected from a culture supernatant of 50–100 μg/ml is more than 100 times greater than that of other VLPs. Secondly, the outcome of gene delivery in humans can be predicted using conventional laboratory animals, since HEV naturally infects various animals as well as humans through the same infectious route and target cells.[10,20] Thirdly, HEV-VLPs are stable at room temperature. Fourthly, anti-HEV immune responses had no effect on DNA administration in the present study, and this might be related to the neutralizing antibody for preventing infection with HEV. Neutralizing antibodies to HEV for inhibiting infection have not yet been found. This is also the case for HCV. The mechanism by which HEV is eliminated by antibodies is thought to be antibody-dependent cell-mediated cytotoxicity (ADCC). The effect of induction of immune responses to DNA vaccine in our system is not clear. Thus, HEV-VLPs are an attractive vaccine vector in developing countries because these VLP can be preserved without the requirement of any particular equipment. Finally, we have reported that an HEV-VLP can carry foreign amino-acid sequences as a part of the ORF2 protein exposed on the particle surface without any morphological or biological alteration.[10] Liposomal vectors resembling retroviral envelopes endowed with targeting molecules for gene delivery have been reported. The vicronectin receptor, $\alpha_v\beta_3$-integrin, is commonly upregulated on malignant melanoma cells, and liposome carrying an Arg-Gly-Asp (RGD) integrin-binding motif has been used for a system to deliver DNA to these tumor cells.[21] It has also been reported that targeting DNA to M cells by intranasal administration for the induction of mucosal and systemic responses can be achieved by formulating DNA with polylysine linked to viral adhesion.[22] It may be possible to design chimeric ORF2 proteins carrying these targeting molecules to retarget HEV-VLP to particular cell types.

Oral vaccination has obvious advantages for a field trial in a large-scale public health vaccination program.[23] From a practical standpoint, oral administration is less stressful for vaccine recipients and does not require professional skill for the vaccine administration. Moreover, delivery of vaccines via the intestinal tract is considered to be inherently safer than systemic injection. Encouraging results of phase I trials using Norwalk virus VLPs have recently been reported.[24] Trials using DNA vaccines for infectious and malignancy diseases have also been conducted.[25] The results of the present study suggest that oral administration of DNA vaccine encapsulated in oral transmissible virus VLPs, HEV-VLPs, is effective for inducing both humoral and cellular immunity locally as well as systematically. HEV-VLPs might be useful not only for vaccination but also as a vector in human gene therapy.

## Materials and methods

### Mice

BALB/c female mice were purchased from Clea Japan (Tokyo, Japan) and were housed in the Laboratory Animal Center of Mie University School of Medicine during the experimental period.

### Peptide synthesis

The peptides used in this study were the HIV env CTL epitope (HIV 308-322, RIQRGPGRAFVTIGK; P18)[26] and a control peptide (HCV nonstructural protein 5 CTL epitope MSYSWTGALVTPCAAE; P17).[27]

### Plasmid DNA

A highly efficient mammalian expression vector, pJW4303,[28] was used for efficient expression of HIV env gp120 of the NL432 strain.[29] Various sizes of plasmid DNA were also used for the *in vitro* packaging experiment (3.162 kb: pUC118; 5.93 kb: pJW322; 8.63 kb: pJWSIVenv; 11.2 kb: pABWN).

### Production and purification of HEV-VLPs

HEV-VLPs were produced and purified by previously described methods.[10,11] Briefly, Tn5 cells maintained in Excel 405 serum-free medium (JRH, KS) were infected with the recombinant baculovirus expressing HEV-ORF2 at an m.o.i. of >5 and cultured for 6 days. The supernatant was harvested and the recombinant baculovirus in the supernatant was pelleted by ultracentrifugation at 10 000 *g* for 30 min at 4°C. The VLPs in the supernatant were collected by further ultracentrifugation at 100 000 *g* for 2 h at 4°C. Pelleted VLPs were then resuspended in 10 mM potassium-[2-(*N*-morpholino) ethanesulfonic acid] (MES) buffer (pH 6.2) and purified on a CsCl equilibrium density gradient. The purified HEV-VLPs were spun down and resuspended in potassium-MES buffer and kept at 4°C.

### DNA packaging

Plasmid DNA was encapsulated into HEV-VLPs according to a previously described procedure.[30] Purified VLPs (50 μg) were disrupted by incubation in 180 μl of a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA and 20 mM dithiothreitol. Following 30 min of incubation at room temperature, 200 μg (20 μl) of each plasmid in 50 mM Tris-HCl buffer (pH 7.5) and 150 mM NaCl was added. The disrupted VLP preparation was refolded by incubation for 1 h with increasing concentrations of $CaCl_2$ up to a final concentration of 5 mM. VLPs were pelleted by ultracentrifugation and resuspended in 10 mM potassium-MES buffer (pH 6.2). At each step, the VLP structure formation was confirmed by electron

microscopy after negative staining, as described previously.[11] To estimate the amounts of encapsulated plasmid DNA, refolded and purified VLPs were treated with 10 IU benzonase (SIGMA-ALDRICH, Irvin, UK) for 1 h at 20°C to remove DNA on the surfaces of VLPs and disrupted with EGTA (1 mM). Absorbance of the supernatant was measured for detection of plasmid DNA contents.

### *Density analysis of refolded VLPs*

Refolded VLPs were separated on a CsCl equilibrium density gradient and fractioned into 0.2 ml aliquots. HEV-VLPs in each fraction were detected by ELISA as previously described,[10] as well as DNA contents.

### *Gene transfer in mammalian cells*

Four cell lines (NIH/3T3 (mouse), RK13 (rabbit), COS-7 (monkey), HepG2 (human)) were used in transfection experiments. Sterilized coverslips were placed in six-well plates, and $5 \times 10^5$ cells per well were seeded in the plates. After overnight culture, cells were washed twice with a medium, and about 1 μg of VLP-encapsulated EGFP expression vector (BD Bioscience Clontech, CA, USA) diluted with 0.5 ml medium was added. After 2 h of incubation at 37°C, VLPs were removed. Cells were then incubated for 48 h at 37°C. At the end of the culture period, cells were removed from the culture medium and washed three times with PBS. Coverslips were then mounted onto microscope slide glasses. Fluorescence of the GFP-expressing cells was observed under a fluorescence microscope.

### *Immunization*

Mice were orally immunized four times with 50 μg protein of HEV-VLP/DNA (pJWNL432) complex or 20 μg naked pJWNL432 DNA in 100 μl of potassium-MES buffer at 1 week intervals.

### *Immunohistochemical analysis*

At 2 days after oral immunization, the mice were killed and tissues were collected. Cryostat sections were air-dried and incubated in 0.5% $HIO_4$ for 10 min to quench endogenous peroxidase activity. The sections were further pretreated with chicken anti-mouse IgG antibody (Chemicon International, Inc., CA, USA) to prevent nonspecific reactions of a secondary antibody. The sections were then incubated with an HIV env-specific mAb (HIV-1 IIIB gp120 mAb (902)), which was obtained through the AIDS Research and Reference Reagent Program,[31] for 30 min at 37°C. The bound antibodies were visualized with a biotinated secondary antibody, HRP-labeled avidin–biotin complex (ABC-peroxidase staining kit, Elite Vector Lab. Inc., CA, USA) and 3.3′-diaminobenzidine tetrachloride with 0.01% $H_2O_2$. Sections were slightly counterstained with hematoxylin. An mAb (A1/3D1, ANOGEN, Canada) against hepatitis C virus core, which is same isotype to 902, was used as a control.

## *ELISA*

Serum and fecal samples were collected at 0 (preimmunization), 2, 4, 6 and 12 weeks after the first immunization. Feces were suspended in ice-cold PBS at 200 mg/ml, and the centrifuge supernatant was used as fecal extract. Culture plates (96-well) were coated with purified HEV-VLPs or synthesized oligopeptides (P18) at a concentration of 10 or 100 μg/well, respectively, overnight at 4°C followed by 30 min of blocking with PBS containing 0.1% FBS and 0.05% Tween 20. To determine the anti-HIV env gp120 antibody responses, CV-1 cells were seeded in 96-well plates and infected with recombinant Sendai virus expressing HIV env gp120 of NL432 strain (SeV gp120),[32] and then the plates were incubated at 37°C. At 3 days after infection, plates were washed and fixed with PBS containing 10% formalin for 10 min. Test samples were added to each well and incubated at room temperature for 1 h. For detection of anti-HIV env gp120 antibody, test samples were reacted with wild-type Sendai virus-infected CV-1 cells before addition to the wells to eliminate the nonspecific antibody. Biotin-labeled anti-mouse IgG (Vector, CA, USA) or IgA (CALTAG, CA, USA) was used as the detection antibody. Following 1 h incubation, the plates were washed and further incubated with avidin-HRP (Vector, CA, USA). The reaction was developed using an ABTS substrate (Roch Diagnostic, Mannheim, Germany).

### *Generation of CTL effector cells*

Effector cells were derived from spleen, MLN and PP cells as precursor CTLs. Aliquots of $5 \times 10^6$ spleen cells were co-cultured with $2.5 \times 10^6$ mitomycin C-treated autologous spleen cells labeled with a peptide at 37°C in a $CO_2$ incubator. The effector cells generated were harvested after 5 days of culture.

### *Cytotoxicity assay*

Target cells, A20.2J cells ($2 \times 10^6$), were incubated at 37°C in a 5% $CO_2$ atmosphere with 10 μg/ml of P18 or control peptide for 16 h. The target cells were then washed and labeled with $^{51}Cr$. The $^{51}Cr$-labeled target cells were incubated for 5 h with effector cells. Spontaneous release varied from 5 to 10%. Percent lysis was calculated as ((experimental release−spontaneous release)/(100% release−spontaneous release)) × 100. All the experiments were performed at least four times, and each experimental group consisted of five mice.

### *Blocking of cytolysis*

$^{51}Cr$-labeled target cells ($10^6$ cells) were preincubated at 4°C for 1 h with anti-H-2 $K^d$, $D^d$ or $L^d$ mAb (Meiji Institute of Health Science Ltd., Tokyo, Japan) (1 μg/ml), and effector cells were then added. In a separate experiment, effector cells ($10^7$ cells) were preincubated with anti-CD4 mAb (GK1.5) or anti-CD8 mAb (Lyt2.2) (10 μg/ml) at 4°C for 1 h, and then the labeled target cells were added. Blocking of cytolytic activities by these mAbs was assessed by a 5-h $^{51}Cr$ release assay.

### *Statistical analysis*

Statistical analysis was performed using Mann–Whitney's U test and Kruskal–Wallis test. Values are expressed as means ± s.d.s. A 95% confidence limit was taken as significant ($P < 0.05$).

## Acknowledgements

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＜速　報＞

# 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

加藤　将[1)]*　種市　幸二[1)]　松林　圭二[2)]

**緒言**：E型肝炎が本邦で最も多発するのは北海道であり，その理由の一つは豚レバーの摂食に帰せしめられている[1)]．最近我々は，北海道内の焼肉店て会食した13名の親類縁者中7名にE型肝炎ウイルス(HEV)感染マーカーを検出するという，集団感染事例を経験したので報告する．

**発端となった症例**：69歳男．2004年9月21日に高度肝機能異常を呈して当科入院．25日に羽ばたき振戦，29日には傾眠が出現し(PTは22%)，30日にIgM anti-HEV陽性所見によりE型肝炎と診断．10月14日に劇症肝炎で死亡．患者家族からの聴き取り調査で，患者及びその親類縁者合計13名が，お盆の8月14日に各々の居住地から帰省し，北見市内の焼肉店で会食していたことが判明した．

**家族の調査**：患者の次男が9月20日に献血．その際の検査でALT＝236，HEV RNA＋．他の家族にも感染者が存在する可能性を疑い，8月14日の会食に参加した全ての家族から採血とアンケート調査を行った．Table 1にその結果を示す．

**HEV RNAの解析**：患者次男から採取したHEVはgenotype IVであった．患者検体に含まれるHEV RNAは極めて低力価であり解析は困難を極めたが，genotype IVであることは辛うじて判明した．

**考案**：我が国のHEV感染の一部がzoonotic food-borne transmissionによるものであることを，既に幾つかの報告が示唆している[1~4)]．しかし，市井の料理店で感染した可能性を示唆する報告は本事例を以て嚆矢とする．感染源となった食材が何であったかを本事例から特定するには至らなかったが，少なくとも豚レバーは本事例でも最も疑わしい食材の一つであった．

**索引用語**：E型肝炎，zoonosis，焼肉，北海道

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## 英文要旨

A mini-outbreak of HEV infection in those who enjoyed *Yakiniku* party：one died of fulminant hepatitis

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A patient died of fulminant hepatitis E in our hospital. To our surprise, a son of the patient was also infected with HEV. By executing a family study, it was revealed that 7 out of 13 relatives of the patient, all of whom had participated in a *Yakiniku* party, had infection markers for HEV. Our results corroborate previous report suggesting pig liver to be an important infection source in Hokkaido, Japan.

*Kanzo* 2004；45：688

**Table 1**　HEV infection markers in the 13 family members who participated in the *Yakiniku*-dinner on 14 Aug 2004

| Case # | Age-sex | Days after 14 Aug '04 | ALT (IU/*l*) | HEV markers | | | Meats consumed on 14 Aug '04* | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | RNA | IgM | IgG | beef | pork | pork liver | *Horumon*** | chicken |
| 1 | 39-m | 37 | 236 | + | + | + | 0 | 2 | 2 | 0 | 2 |
| 2(index) | 69-m | 41 | 1511 | + | + | + | NA | NA | NA | NA | NA |
| 3 | 68-m | 79 | 15 | − | + | + | 2 | 2 | 0 | 2 | 0 |
| 4 | 37-f | 79 | 13 | − | + | + | 2 | 2 | 0 | 2 | 0 |
| 5 | 15-m | 90 | 17 | − | + | + | 2 | 2 | 2 | 1 | 1 |
| 6 | 43-m | 92 | 34 | − | + | + | 2 | 2 | 1 | 2 | 1 |
| 7 | 58-f | 79 | 25 | − | − | + | 2 | 2 | 0 | 2 | 0 |
| 8 | 15-m | 77 | 19 | − | − | − | 2 | 0 | 0 | 0 | 2 |
| 9 | 14-m | 77 | 19 | − | − | − | 2 | 0 | 0 | 2 | 0 |
| 10 | 67-f | 79 | 15 | − | − | − | 0 | 1 | 0 | 0 | 0 |
| 11 | 38-f | 89 | 12 | − | − | − | 1 | 0 | 0 | 1 | 0 |
| 12 | 46-m | 90 | 15 | − | − | − | 1 | 1 | 0 | 0 | 0 |
| 13 | 6-f | 90 | 15 | − | − | − | 1 | 0 | 0 | 1 | 1 |

* 0＝not eaten；1＝eaten a little；2＝eaten much；and NA＝information not available.** Supposed to be "rectum of pig"

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*Case report*

# Three male patients with sporadic acute hepatitis E in Sendai, Japan, who were domestically infected with hepatitis E virus of genotype III or IV

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Recent studies indicate that hepatitis E virus (HEV) infection occurs not only in developing countries but also in industrialized nations. However, the characteristics of domestic infections of hepatitis E in Japan are not fully understood. We analyzed serum samples from 34 patients who were seen at a city hospital in Sendai, Japan, between January 1997 and December 2002, and who had been given the diagnosis of sporadic acute hepatitis of non-A, non-B, non-C etiology. Among these 34 patients, 3 (9%; all men; aged 54, 59, and 61 years) were positive for both IgG and IgM anti-HEV antibodies and for HEV RNA. The HEV isolates (HE-JAS1 and HE-JAS3) obtained from case 1 and case 3, respectively, segregated into genotype III; they had the highest nucleotide sequence identity, of 99.5% and 99.0%, with HE-JA7 and HE-JA8, respectively, both of which had been isolated in Iwate, a neighboring prefecture of Sendai. In contrast, the remaining HEV isolate (HE-JAS2), obtained from case 2, segregated into genotype IV; it had the highest nucleotide sequence identity, of 99.8% and 99.3%, with JKK-Sap and HE-JA3, respectively, both of which had been isolated in Hokkaido, Japan, although case 2 had never been to Hokkaido. Our three patients with hepatitis E had not traveled abroad in the preceding 1 year, had had no contact with pigs, and no history of blood transfusion. These results indicate that HEV should be considered as an etiological agent of acute hepatitis of non-A, non-B, non-C etiology in Japan. The risk factor(s) for acquiring domestic HEV infection in Japan needs to be clarified in future studies.

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The nucleotide sequences of the 3 HEV isolates reported herein have been assigned DDBJ/EMBL/GenBank accession nos. AB107366-AB107368.

**Key words:** acute hepatitis E, domestic infection, genotype, hepatitis E virus, phylogenetic tree

## Introduction

Hepatitis E virus (HEV), the major etiological agent of enterically transmitted non-A, non-B hepatitis, was previously considered to be endemic only in developing countries with poor sanitation, including countries in Asia, Africa, and Latin America.[1] Recently, however, isolated patients with hepatitis E, with no history of travel to areas endemic for hepatitis E, have been reported in industrialized countries.[1–4] The genome of HEV is a single-stranded, positive-sense RNA of approximately 7.2kb, and contains a short 5′ untranslated region (UTR), three open reading frames (ORFs; ORF1, ORF2, and ORF3) and a short 3′ UTR terminated by a poly(A) tract.[5,6] Based on sequence analysis, HEV isolates have been classified into four major genotypes (genotypes I–IV).[3] HEV isolates from patients with sporadic cases of HEV infection in industrialized countries were found to belong to genotypes III and IV, and are distinct from HEV isolates from patients in developing countries (genotype I in Asia and Africa and genotype II in Mexico and Nigeria).[3]

In Japan, multiple HEV strains of genotype III or IV have been recovered from patients with sporadic acute or fulminant hepatitis E who had not traveled abroad.[7–11] However, the clinical features of domestic HEV infection, as well as the serological and molecular characteristics of, presumably, Japan-indigenous HEV strains have not been fully elucidated. Furthermore, the mode of HEV transmission in isolated cases of hepatitis E in Japan is not known. Therefore, to shed some light on the clinical, epidemiological, and virological characteristics of HEV infection in industrialized countries, including Japan, we report three cases of sporadic acute

hepatitis E in patients who lived in Sendai, Japan, and who had not traveled outside Japan in the preceding 1 year.

## Subjects, materials, and methods

### *Serum samples*

Serum samples were obtained on admission from 34 patients (14 males and 20 females; age, 46 ± 13 years [mean ± SD]) who were seen at Tohoku Rosai Hospital between January 1997 and December 2002, with symptoms such as general fatigue, fever, nausea, and diarrhea, and who had been given the diagnosis of sporadic acute hepatitis of non-A, non-B, non-C etiology. No patient was positive for anti-hepatitis A virus (anti-HAV) IgM (RIA; Dainabot, Tokyo, Japan), hepatitis B surface antigen (RIA or CLIA; Dainabot), anti-hepatitis B core IgM antibody (RIA; Dainabot), or anti-hepatitis C virus antibody (RIA; Dainabot). These patients had no serological markers of ongoing infection with Epstein-Barr virus (EBV), cytomegalovirus (CMV), or herpes simplex virus. There was no evidence of autoimmune liver disease, alcoholic liver disease, drug-induced liver disease, or metabolic disease. Serum samples that had been obtained on admission, with the patient's consent, were kept at −70°C until subsequent testing.

### *Detection of anti-HEV antibody*

IgG-class and IgM-class anti-HEV antibodies were assayed by enzyme-linked immunosorbent assay (ELISA), using purified recombinant ORF2 protein according to the method described previously.[10] To test for anti-HEV IgG or anti-HEV IgM, serum samples were subjected to ELISA, at a dilution of 1:100 in saline, containing 40% (vol/vol) calf serum (GIBCO-BRL, Grand Island, NY, USA). An optical density (OD) of 0.152 for anti-HEV IgG antibody, and that of 0.353 for anti-HEV IgM antibody were used as the cut-off values.[10] The relative titers of anti-HEV IgG or anti-HEV IgM antibodies were estimated by testing additional dilutions (1:1000 and 1:10000) of the serum.

### *Detection of HEV RNA and sequence analysis*

Total RNA that had been extracted from the patient's serum sample with Trizol LS Reagent (Invitrogen, Groningen, Netherlands) was reverse transcribed using SuperScript II RNase H reverse transcriptase (Invitrogen). Using primers specific for the HEV ORF2 sequence, nested polymerase chain reaction (PCR) was performed as described previously.[10] The PCR products were electrophoresed on an agarose gel, stained with ethidium bromide, and photographed under ultraviolet light. The amplification products were sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Sequence analysis was performed using Genetyx-Mac version 12.0.2 (Software Development, Tokyo, Japan) and ODEN version 1.1.1 from the DNA Data Bank of Japan (DDBJ; National Institute of Genetics, Mishima, Japan).[12] Phylogenetic trees were constructed by the neighbor-joining method,[13] based on the partial nucleotide (nt) sequence of the ORF2 region (412nt). The final tree was obtained using the TreeView program (version 1.6.6).[14]

## Results

Among the 34 patients who had been given the diagnosis of acute hepatitis of non-A, non-B, non-C etiology, 8 patients (24%) were positive for anti-HEV IgG antibody, with an OD value ranging from 0.205 to more than 3.000. Of these 8 patients, 3 patients (9%; 3/34) were also positive for anti-HEV IgM antibody, with an OD value ranging from 2.455 to more than 3.000, and

**Table 1.** Profiles of the three patients who contracted sporadic acute hepatitis E

| Case no. | Age (years)/ sex | Date of onset | Peak T. Bil (mg/dl)[a] | Peak ALT (IU/l)[a] | Peak AST (IU/l)[a] | Prothrombin time (%)[b] | OD at 450nm[c] IgM-class anti-HEV | OD at 450nm[c] IgG-class anti-HEV | Name of HEV isolate | HEV genotype |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 61/Male | Mar. 17, 1997 | 12.7 | 1287 | 1039 | 84.6 | >3.000 | >3.000 | HE-JAS1 | III |
| 2 | 54/Male | Jan. 22, 2001 | 10.1 | 5620 | 4590 | 43.1 | >3.000 | >3.000 | HE-JAS2 | IV |
| 3 | 59/Male | Jan. 27, 2001 | 3.4 | 2547 | 1327 | 88.9 | 2.540 | 2.311 | HE-JAS3 | III |

OD, optical density; HEV, hepatitis E virus
[a]T. Bil, total bilirubin (normal range [NR], 0.3–1.2mg/dl); ALT, alanine aminotransferase (NR, 7–34IU/l); and AST, aspartate aminotransferase (NR, 12–33IU/l)
[b]Lowest value during the follow-up
[c]Detected on admission

had detectable HEV RNA (Table 1). Consequently, these three patients (cases 1–3) were retrospectively diagnosed with acute hepatitis E.

*Case 1*

Case 1 was a 61-year-old Japanese man who was hospitalized on March 21, 1997. He worked in the office of a bus company, lived with his wife, and had no pet animals. He had not traveled outside Japan in the preceding 1 year. He was a heavy drinker and had consumed over 80 g of ethanol daily for 43 years; he used to drink alcohol at Japanese-style bars. His initial symptom, dark urine, had appeared 4 days prior to admission. Physical examination on admission was essentially normal, except for jaundice. Laboratory data on admission showed an elevated total bilirubin (T. Bil) level, of 3.6 mg/dl (normal range, 0.3–1.2 mg/dl); aspartate aminotransferase (AST) level of 1039 IU/l (normal range, 12–33 IU/l), alanine aminotransferase (ALT) level of 1072 IU/l (normal range, 7–34 IU/l); alkaline phosphatase (ALP) level of 901 IU/l (normal range, 103–302 IU/l); and γ-glutamyl transpeptidase (γ-GTP) level of 564 IU/l (normal range, 5–57 IU/l). Laparoscopy performed 11 days after admission revealed an icteric green liver with a smooth surface. A liver needle biopsy showed cholestasis, with bile plugs in the canaliculi, infiltration of numerous inflammatory cells in the portal tract, and focal necrosis scattered throughout the parenchyma, although the lobular architecture was preserved. These features were consistent with the findings of acute hepatitis. He had a prolonged episode of jaundice lasting for 35 days after the onset of the illness, but he recovered and was discharged on April 23, 1997 (see Fig. 1 for clinical course of T. Bil, ALT, and AST levels).

*Case 2*

Case 2 was a 54-year-old Japanese man who visited an Internal Medicine Clinic with a chief complaint of general malaise on January 27, 2001, 5 days after the onset of the illness. He was soon transferred to our hospital because of markedly elevated levels of T. Bil (7.4 mg/dl), ALT (5170 IU/l), and AST (5084 IU/l) and a prolonged prothrombin time, of 44%. He worked as a businessman at a food company, lived alone, with no pets, and used to eat out. His last trip abroad had been about 20 years previously. On admission, physical examination revealed jaundice, and his laboratory data were as follows: T. Bil, 9.2 mg/dl; ALT, 5620 IU/l; AST, 4590 IU/l; ALP, 1089 IU/l; and γ-GTP, 818 IU/l; and prothrombin time of 43.1%. The maximum severity of the illness occurred on admission. After admission, he recovered rapidly and was discharged on the twenty-sixth hospital

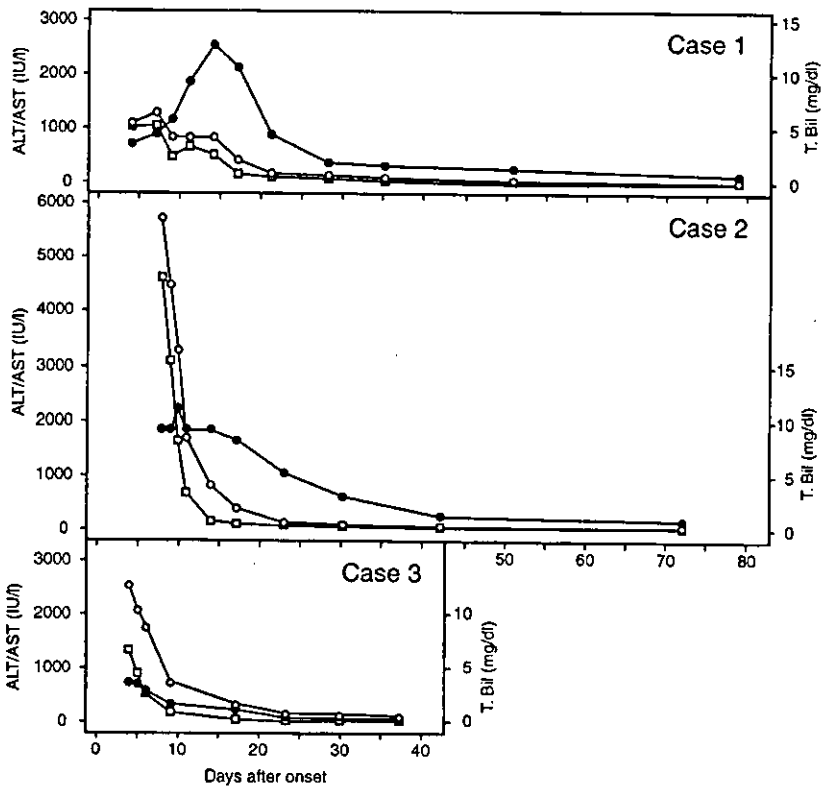


**Fig. 1.** Clinical courses of sporadic acute hepatitis E. The alanine aminotransferase (*ALT; open circles*), aspartate aminotransferase (*AST; open squares*), and total bilirubin (*T. Bil; closed circles*) levels in the initial and follow-up serum samples from three patients with hepatitis E (*cases 1–3*) are shown

day (see Fig. 1 for clinical course of T. Bil, ALT, and AST levels).

*Case 3*

Case 3 was a 59-year-old Japanese man who was admitted with general fatigue and dark urine on January 31, 2001. He was a local civil servant living with his wife and a pet cat. He had drunk beer, at 750 to 1000ml per day, for 35 years. He had traveled around Europe 4 years before admission. Physical examination was normal and laboratory data were as follows: T. Bil, 3.4mg/dl; AST, 1327IU/l; ALT, 2547IU/l; ALP, 722IU/l; γ-GTP, 747IU/l; and prothrombin time of 88.9%. Laparoscopy performed 13 days after admission revealed a slightly icteric liver, and liver needle biopsy showed findings consistent with the recovery stage of acute hepatitis. He recovered rapidly without receiving any medication for acute hepatitis, and was discharged on March 6, 2001 (see Fig. 1 for clinical course of T. Bil, ALT, and AST levels).

*HEV isolates obtained from the three patients in the current study*

The three HEV strains isolated from cases 1–3 in the present study were designated as HE-JAS1, HE-JAS2, and HE-JAS3, respectively. HE-JAS1 and HE-JAS3 were classifiable into genotype III, and HE-JAS2 was classifiable into genotype IV. When the 412-nt ORF2 partial sequences of these three isolates were compared with those of reported HEV isolates of Japan and non-Japan origin, HE-JAS1 had a high nucleotide (nt) sequence identity, of 99.5%, with HE-JA7,[10] and HE-JAS3 had a high nucleotide sequence identity, of 99.0%, with HE-JA8.[10] HE-JAS2 had 99.8% and 99.3% identities with JKK-Sap[8] and HE-JA3,[10] respectively. A phylogenetic tree, constructed based on the 412-nt sequence of the ORF2 region, confirmed that the three HEV isolates obtained in the present study were closely related to Japan-indigenous HEV isolates of genotype III or IV (Fig. 2). Of interest, these three Sendai isolates differed by 17.5%–20.7% from the first Sendai isolate, named HEV-Sendai (AB093535), that had been obtained from a 54-year-old patient who developed sporadic acute hepatitis E in Sendai in June 2002 and who had never traveled abroad,[15] indicating that multiple HEV strains are circulating in the Sendai area.

*Relative titers of anti-HEV IgG and IgM antibodies*

In the serum samples obtained during the acute phase (6 to 8 days after the onset of hepatitis E), both IgG and IgM classes of anti-HEV were detected at the dilution of 1:10000 in case 1. However, anti-HEV IgM was not detectable at the dilution of 1:10000 in case 2, and anti-HEV IgG and anti-HEV IgM were not detectable at the dilutions of 1:10000 and 1:1000, respectively, in case 3 (Table 2). In the follow-up serum samples obtained 2.0 to 5.8 years after the disease onset, anti-HEV IgM was not detected in the sera from any of the three patients at the dilution of 1:100, in line with our expectations. In the serum from case 1, the OD value of anti-HEV IgG was only 0.418 at the dilution of 1:100, and it was below the cutoff value at the dilution of 1:1000. Similarly, in the serum from case 3, the OD value of anti-HEV IgG was 0.704 at the dilution of 1:100, but it was below the cutoff value at the dilution of 1:1000. In contrast, in the

**Table 2.** Relative titers of IgG-class and IgM-class anti-HEV antibodies in serum samples obtained from the three present cases and two previously reported cases of sporadic acute hepatitis E

| Case no. | Age (years) | Days (years) after onset | OD value of anti-HEV in serum at a dilution of: IgG-class 1:100 | IgG-class 1:1000 | IgG-class 1:10000 | IgM-class 1:100 | IgM-class 1:1000 | IgM-class 1:10000 |
|---|---|---|---|---|---|---|---|---|
| 1 | 61 | 7 | **>3.000**[a] | **2.599** | **1.262** | **>3.000** | **2.445** | **0.567** |
| | 66 | 2130 (5.8) | **0.418** | 0.057 | — | 0.074 | — | — |
| 2 | 54 | 8 | **>3.000** | **1.900** | **0.346** | **>3.000** | **1.364** | 0.218 |
| | 56 | 723 (2.0) | **>3.000** | **0.524** | 0.064 | 0.316 | — | — |
| 3 | 59 | 6 | **2.311** | **0.477** | 0.037 | **2.540** | 0.328 | — |
| | 61 | 725 (2.0) | **0.704** | 0.100 | — | 0.070 | — | — |
| A[b] | 40 | 9 | **>3.000** | **1.745** | **0.228** | **>3.000** | **0.735** | 0.104 |
| | 40 | 145 | **>3.000** | **1.948** | **0.268** | **0.754** | 0.075 | — |
| | 48 | 3186 (8.7) | **>3.000** | **0.369** | 0.044 | 0.138 | — | — |
| B[c] | 38 | 0 | **>3.000** | **1.386** | 0.147 | **1.682** | 0.239 | — |
| | 55 | 6373 (17.4) | **0.253** | 0.029 | — | 0.033 | — | — |

[a] OD value that is equal to or greater than the cutoff value is indicated in boldface type
[b] Reported case of a Japanese woman who had never traveled abroad and who developed sporadic acute hepatitis in 1993[18]
[c] Reported case of a Japanese man who had not traveled outside Japan and who contracted sporadic acute hepatitis in 1982[19]

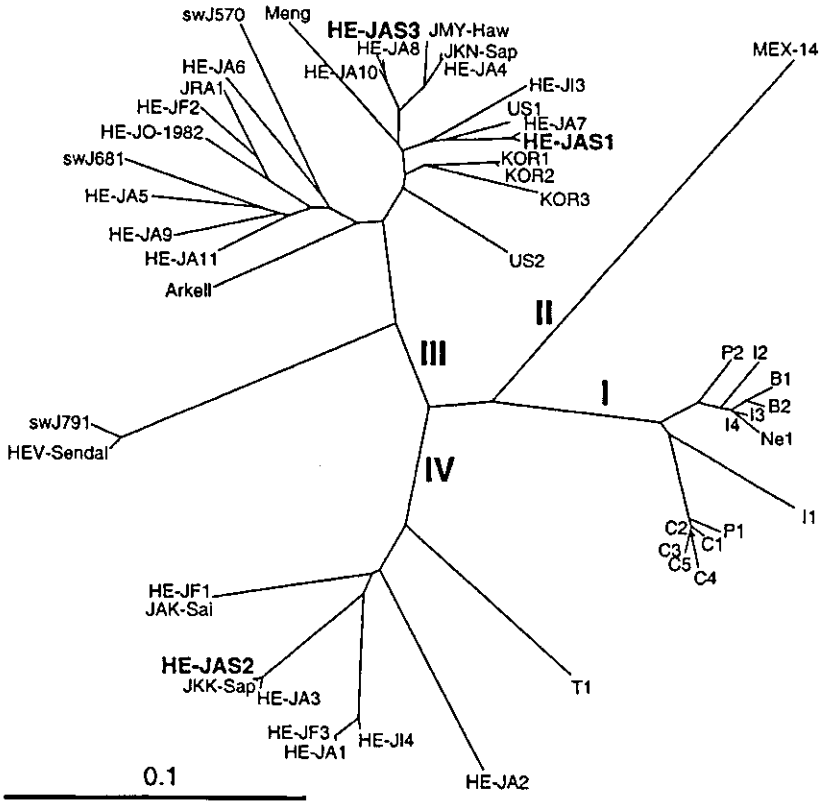


**Fig. 2.** Phylogenetic tree, constructed by the neighbor-joining method, based on the partial nucleotide (nt) sequence (412nt) of the open reading frame (ORF)2 region of 52 hepatitis E virus (HEV) isolates of Japan and non-Japan origin. In addition to 28 reported human and swine HEV isolates of genotypes I–IV whose entire or nearly entire sequence is known, 21 reported isolates of genotype III or IV whose partial sequence of 412, 421, or 860nt has been determined, were included for comparison. The HE-JAS1, HE-JAS2, and HE-JAS3 isolates obtained in the present study are indicated in *boldface type* for visual clarity. The accession numbers of the reported 28 isolates whose entire or nearly entire sequence is known are as follows: M73218 (B1), D10330 (B2), D11092 (C1), L25595 (C2), M94177 (C3), D11093 (C4), L08816 (C5), X98292 (I1), X99441 (I2), AF076239 (I3), AF459438 (I4), AF051830 (Ne1), AF444003 (P1), and AF185822 (P2) of genotype I; M74506 (MEX-14) of genotype II; AF060668 (US1), AF060669 (US2), AF082843 (Meng), AP003430 (JRA1), AB073912 (swJ570), AB074918 (JKN-Sap), AB074920 (JMY-Haw), AB089824 (HE-JA10), and AY115488 (Arkell) of genotype III; and AJ272108 (T1), AB074915 (JAK-Sai), AB074917 (JKK-Sap), and AB080575 (HE-JI4) of genotype IV. The accession numbers of the reported 21 isolates whose partial sequence of 412–1497nt is known are as follows: AB073910, AB073911, AB079762–AB079764, AB080579, AB088418, AB082557–AB082565, AB082567, AB093535, AF516178, AF516179, and AF527942. Genotype numbers I–IV are in accordance with the recent report by Schlauder and Mushahwar.[3] Eight HEV isolates of Meng, Arkell, swJ570/681/791, and KOR1/2/3 were recovered from pigs in the United States, Canada, Japan, and Korea, respectively

serum from case 2, the OD value of anti-HEV IgG was more than 3.000 at the dilution of 1:100, and it was still above the cutoff value at the dilution of 1:1000.

## Discussion

In the present study, 3 (9%) of 34 patients who had been given the diagnosis of sporadic acute hepatitis of non-A, non-B, non-C etiology, were positive for anti-HEV IgG, anti-HEV IgM, and HEV RNA, and were diagnosed with hepatitis E. Among the remaining 31 patients with acute hepatitis of unknown etiology, 5 patients (16%) had anti-HEV IgG antibody alone, with an OD value of 0.205, 0.209, 0.248, 0.337, or 0.977, representing past infection of HEV. Although the OD value of anti-HEV IgG in these patients was only 1.3- to 6.4-fold the cutoff value, reproducibility of the antibody assay was confirmed. In addition, the specificity of the assay was assessed as follows. The OD value of the five samples decreased to less than 30% of the original value after absorption with the same recombinant ORF2 protein that was used as the antigen probe, but it remained greater than 90% of the original

value after absorption with a mock protein obtained from the pupae of silkworm infected with nonrecombinant baculovirus. One patient with anti-HEV IgG alone had traveled to Australia 5 years earlier, but the remaining 4 patients had never traveled outside Japan, suggesting domestic infection of HEV in Japan. The prevalence of anti-HEV IgG antibody has been reported to be 1.9%–14.1% in Japan, differing by geographical region.[16] Therefore, a seroepidemiological survey of HEV infection in the Sendai area is needed in future studies, to clarify the region-dependent endemicity of hepatitis E, as was conducted in Nagano prefecture, Japan.[17]

In our previous studies,[18,19] the anti-HEV IgG level was high in the acute phase of hepatitis E and then gradually decreased, although it remained at a high titer even 8.7 years after the onset of hepatitis E, and still tested positive 17.4 years after the disease onset (Table 2). In case 3 in the present study, however, the anti-HEV IgG level was relatively low in the acute-phase serum, and the OD value at a dilution of 1:100 was only 0.704 at 2 years after the onset of the disease. In contrast, although the anti-HEV IgG level was relatively high in the acute-phase serum of case 1, the OD value at a dilution of 1:100 was only 0.418 at 5.8 years after the disease onset, indicating a rapid reduction in the anti-HEV IgG level. These results suggest that the extent of the antibody response to HEV differs markedly among individuals who contract clinical HEV infection, and, therefore, it is difficult to estimate the precise time at which a patient acquired HEV infection from the anti-HEV IgG level in the recovery-phase serum.

The HEV isolates obtained from case 1 (HE-JAS1) and case 3 (HE-JAS3) in the current study were of genotype III, and were most closely related to HE-JA7 (99.5% identity) and HE-JA8 (99.0% identity), respectively, both of which had been isolated from patients with sporadic acute hepatitis E who lived in Iwate prefecture in Japan.[10] Of note, in our previous study,[10] 4 (18%) of 22 patients with acute hepatitis of non-A, non-B, non-C etiology in Iwate were diagnosed with hepatitis E; Iwate had the second highest prevalence of clinical HEV infection next to Hokkaido (25%; 4/16). Because Iwate prefecture is located north of and adjacent to Miyagi prefecture, where Sendai City is located, it is likely that closely related HEV strains of genotype III are circulating in the two neighboring prefectures. The HEV isolate obtained from case 2 (HE-JAS2) segregated into genotype IV, and was closest to JKK-Sap and HE-JA3, both of which had been isolated from a patient with sporadic acute hepatitis E living in Hokkaido who had no history of travel abroad.[8,10] However, case 2 had never traveled to Hokkaido. Furthermore, cases 1, 2, and 3 had not traveled outside Sendai City in the preceding 2 months, suggesting that our three patients acquired acute hepatitis E in Sendai City.

Although risk factors for acquiring sporadic hepatitis E were not recognized in our patients in the present study, it is probable that the following forms of transmission occur in Japan where sanitation systems have been established: (a) zoonotic infection, (b) transmission by food, (c) person-to-person transmission, and (d) transmission via blood transfusion. It has been reported that person-to-person transmission of HEV during epidemics in HEV-endemic countries plays a minor role in the spread of hepatitis.[20] As to the present cases, the examined family members (cases 1 and 3) were exclusively negative for anti-HEV IgG, indicating that person-to-person transmission of HEV may be extremely rare, if it occurs at all, in Japan. Although viremic blood donors can potentially cause transfusion-associated hepatitis E,[21] none of our patients had received a blood transfusion.

Evidence is accumulating that hepatitis E is a zoonosis,[2,4,22,23] and cross-species infection of HEV has been documented.[24–26] Several recent studies have indicated that in a particular geographical region, HEVs from pigs and humans are closely related genetically,[27–31] further supporting the hypothesis that hepatitis E is a zoonosis. However, none of our patients with hepatitis E in the current study had had any contact with pigs or other animals, such as rats, mice, dogs, cows, sheep, and goats, that could also potentially serve as reservoirs,[1,4] except for a pet cat, in case 3. Therefore, we cannot rule out the possibility that case 3 was infected with HEV from his pet cat.

As described in previous reports,[32,33] it is likely that foods such as raw shellfish act as vehicles for the transmission of HEV. Although no case of hepatitis E has been unequivocally shown to be due to food consumption, we cannot deny the possibility of infection via HEV-contaminated foods in cases 1 and 2, who used to eat out, because undercooked pig livers or intestines/colons are occasionally consumed as barbecued dishes at Korean restaurants or Japanese-style bars, particularly by middle-aged men who are drinking alcohol in Japan, consistent with our previous observation that the prevalence of clinical HEV infection is high among males and among those over 40 years of age.[10]

In conclusion, we encountered three male patients with sporadic acute hepatitis E in Sendai, Japan, who were domestically infected with HEV of genotype III or IV. Because it is unequivocal that HEV is circulating in Japan, when clinicians are confronted with patients with sporadic acute hepatitis of non-A, non-B, non-C etiology, the possibility of the presence of clinical HEV infection should be taken into consideration. Further studies are required to clarify whether domestic HEV infection in Japan is acquired via zoonosis and/or foods.

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# Serological Markers of Hepatitis B, C, and E Viruses and Human Immunodeficiency Virus Type-1 Infections in Pregnant Women in Bali, Indonesia

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Except for hepatitis B virus (HBV), there have been few data on serological markers of hepatitis viruses such as hepatitis C virus (HCV) and E virus (HEV), and human immunodeficiency virus type-1 (HIV) in Bali, Indonesia. During 5 months from April to August 2003, sera were collected from 2,450 pregnant women at eight jurisdictions in Bali, and they were tested for markers of these viruses. Only one (0.04%) was positive for antibody to HCV, but none for antibody to HIV. Hepatitis B surface antigen (HBsAg) was detected in 46 (1.9%) at a prevalence significantly lower than that in 271 of the 10,526 (2.6%) pregnant women in Bali surveyed 10 years previously ($P < 0.045$). The prevalence of hepatitis B e antigen in pregnant women with HBsAg decreased, also, from 50% to 28% during the 10 years ($P < 0.011$). Antibody to HEV (anti-HEV) was examined in 819 pregnant women who had been randomly selected from the 2,450. The overall prevalence of anti-HEV was 18%, and there were substantial regional differences spanning from 5% at Tabanan district to 32% at Gianyar district. Furthermore, the prevalence of anti-HEV differed substantially by their religions. In the Sanglah area of Denpasar City, for instance, anti-HEV was detected in 20 of the 102 (20%) Hindus, significantly more frequently than in only 2 of the 101 (2.0%) Muslims ($P < 0.001$). Swine that are prohibited to Muslims, therefore, is likely to serve as a reservoir of HEV in Bali. In conclusion, HBV is decreasing, HCV and HIV have not prevailed, as yet, while HEV is endemic probably through zoonotic infection in Bali. ***J. Med. Virol. 75:499–503, 2005.*** 

**KEY WORDS:** Bali; hepatitis B e antigen; hepatitis B virus; hepatitis C virus; hepatitis E virus; human immunodeficiency virus type-1; pregnancy; zoonosis

## INTRODUCTION

Bali is an island in Southeast Asia, between the Bali sea and the Indian Ocean, and has approximately 3 million inhabitants. The prevalence of infection with hepatitis C virus (HCV) has not been examined, as yet, although there are a few reports on serological markers of hepatitis B virus (HBV) and hepatitis E virus (HEV) in Bali [Brown et al., 1985; Wibawa et al., 2004]. Nor is it known whether the population in Bali is affected by human immunodeficiency virus type-1 (HIV), except in commercial sex workers [Ford et al., 2000].

Since 1993, pregnant women in Bali have been tested for hepatitis B surface antigen (HBsAg) in serum, and those positive for HBsAg were examined further for hepatitis B e antigen (HBeAg). Babies born to pregnant women carrying HBsAg along with HBeAg have received the passive and active immunoprophylaxis with hepatitis B immune globulin and vaccine [Tada et al., 1982]; it is found highly efficacious in preventing the persistent HBV carrier state in high-risk babies in Japan [Noto et al., 2003].

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Taking advantage of routine screening for HBsAg of pregnant women in Bali, sera were tested for serological markers of HBV, HCV, HEV, and HIV. The results highlighted decreasing HBV infection, rare infection with HCV and HIV, and a high exposure to HEV that depends on habits and religions of the Balinese.

## MATERIALS AND METHODS

### Pregnant Women in Bali

During 5 months from April to August 2003, sera were obtained from 2,450 pregnant women, at major hospitals in the eight jurisdictions of Bali (Fig. 1), on routine surveys for HBsAg for preventing the perinatal transmission of HBV. Their mean age was $27 \pm 5$ (SD) years (range: 16–45 years). The sera were tested for HBsAg, antibody to HCV (anti-HCV) and antibody to HEV (anti-HEV), as well as antibody to HIV (anti-HIV). HBeAg was examined only in sera positive for HBsAg. Sera from all pregnant women were tested for serological markers of these viruses, except for anti-HEV which was examined in approximately 100 each randomly selected in the eight jurisdictions. Anti-HEV was tested in an additional 90 sera from Muslim pregnant women living in the Sanglah area of Denpasar City, in an attempt to find any differences in the prevalence between Hindus and Muslims. The design of the serological survey was in accord with the 1975 Declaration of Helsinki, and approved by Ethics Committee of institutions. Every pregnant woman gave an informed consent.

### Serological Tests for Markers of HBV, HCV, HEV, and HIV

HBsAg was tested by hemmaglutination and immunochromatography (Entebe HBsAg RPHA and Entebe HBsAg Strip, respectively: Hepatika Laboratory, Mataram, Indonesia) and HBeAg by enzyme-linked immunosorbent assay (ELISA) (HBeAg ELISA: Institute of Immunology, Tokyo, Japan). Anti-HCV was determined by the dipstick method (Entebe Anti-HCV Dipstick: Hepatika Laboratory). Anti-HEV of IgG class was determined by ELISA with use of a recombinant HEV capsid protein of genotype IV by the method of Mizuo et al. [2002], and anti-HIV by immunochromatography (Entebe Anti-HIV Strip: Hepatika Laboratory).

HEV RNA was determined by the polymerase chain reaction with primers deduced from the nucleotide sequences in the open reading frame 2 that are preserved irrespective of genotypes [Mizuo et al., 2002].

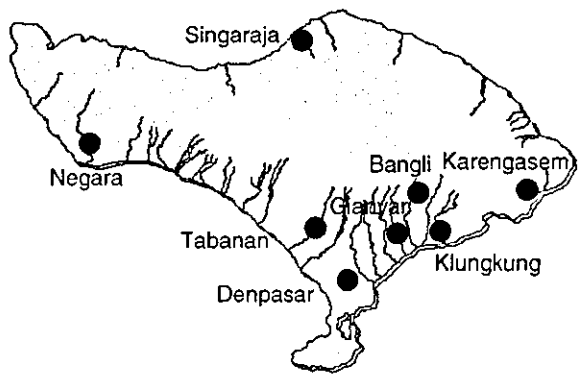


Fig. 1. Map of Bali with eight districts where markers of hepatitis viruses and HIV among pregnant women were surveyed.

### Statistical Analyses

Categorical variables were compared between groups by the Chi-square test, and continuous variables by the Welch's $t$-test. Differences with a $P$ value $<0.05$ were considered significant.

## RESULTS

### HBsAg in Pregnant Women in Bali

Frequencies of HBsAg, anti-HCV, anti-HEV, and anti-HIV in the eight jurisdictions in Bali are listed in Table I. Overall, HBsAg was detected in 46 of the 2,450 (1.9%) pregnant women during 5 months from April to August 2003. This prevalence of HBsAg was significantly lower than that in 271 of the 10,526 pregnant women in Bali surveyed 10 years before in 1993 (1.9% vs. 2.6%, $P < 0.045$).

The prevalence of HBsAg in Negara in the west (Fig. 1) was by far the highest at 4.5% (6/132), in remarkable contrast to 0.6% (1/161) in Tabanan and 0.8% (1/133) in Singaraja. Differences fell short of being significant, however, due to low numbers of pregnant women examined.

Figure 2 illustrates age-specific frequencies of HBsAg and HBeAg. The prevalence of HBsAg stayed constant in a range from 1.6% to 2.5%, while HBeAg decreased with age; it was most frequent in pregnant women aged younger than 25 years (53% [8/15]). Of 46 pregnant women who carried HBsAg, the 13 with HBeAg in serum were significantly younger than the 33 without HBeAg ($24 \pm 4$ vs. $29 \pm 6$ years, $P < 0.0190$).

### Anti-HCV and Anti-HIV in Pregnant Women in Bali

Infection with HCV or HIV was very infrequent in pregnant women in Bali. Anti-HCV was detected in a single pregnant woman in Denpasar, while anti-HIV was not in any. Thus, the prevalence of anti-HCV was 0.04% and that of anti-HIV less than 0.04%.

### Anti-HEV in Pregnant Women in Bali

Anti-HEV was examined in all the 41 pregnant women from Karangasem, and 86–196 randomly selected among those from the other districts. Anti-HEV was detected in 151 of these 819 (18%) pregnant women, producing an overall prevalence of 18%. The mean absorbancy in ELISA on the 151 sera positive for anti-HEV was low at $0.79 \pm 0.61$. HEV RNA was not detected in any of the 20 sera with a high absorbancy (>1.50).

There were marked regional differences in the prevalence of anti-HEV. It was low in Tabanan (4.7% [4/86])

TABLE I. Serological Markers for HBV, HCV, HEV, and HIV Infections in the Eight Jurisdictions of Bali

| Jurisdictions | HBsAg | Anti-HCV | Anti-HEV | Anti-HIV |
|---|---|---|---|---|
| Bangli | 2/115 (1.7%) | 0/115 | 25/93 (27%) | 0/115 |
| Denpasar | 29/1,594 (1.8%) | 1/1,594 (0.06%) | 35/196 (18%) | 0/1,594 |
| Gianyar | 3/151 (2.0%) | 0/151 | 32/101 (32%) | 0/151 |
| Karangasem | 1/41 (2.4%) | 0/41 | 6/41 (15%) | 0/41 |
| Klungkung | 3/123 (2.4%) | 0/123 | 19/98 (19%) | 0/123 |
| Negara | 6/132 (4.5%) | 0/133 | 11/100 (11%) | 0/133 |
| Singaraja | 1/133 (0.8%) | 0/132 | 19/104 (18%) | 0/132 |
| Tabanan | 1/161 (0.6%) | 0/161 | 4/86 (4.7%) | 0/161 |
| Total | 46/2,450 (1.9%) | 1/2,450 (0.04%) | 151/819 (18%)[a] | 0/2,450 (<0.04%) |

[a]Anti-HEV was examined in only 819 samples, randomly extracted from among inhabitants from each jurisdiction, except for Karangasem all pregnant women from where were examined.

and high in Gianyar (32% [32/101]) and Bangli (27% [25/93]); the difference between Tabanan and Gianyar was statistically significant ($P < 0.0001$). Frequencies of anti-HEV in the other five districts were much the same and ranged from 11% to 19%. There were no differences in the mean age among pregnant women from distinct religions.

The prevalence of anti-HEV differed with regard to the religion of the pregnant women (Table II). Overall, anti-HEV was detected in 149 of the 769 (19%) Hindus, at a frequency significantly higher ($P < 0.012$) than that in two of the 50 (4.0%) non-Hindus (mostly Muslims). The frequency of anti-HEV higher in Hindus than non-Hindus held in pregnant women from all the eight jurisdictions. In Denpasar where more women were examined than the other seven districts, anti-HEV occurred more often in Hindus than non-Hindus (19% [33/175] vs. 9.5% [2/21]); the difference fell short of being significant due to small numbers examined.

For evaluating the influence of religions on HEV infection, pregnant women living in the Sanglah area of Denpasar City were examined for the prevalence of anti-HEV; inhabitants in this narrow area were surveyed in an attempt to exclude environmental factors such as water quality and sanitation. Anti-HEV was significantly more frequent in Hindus than Muslims there (20% [20/102] vs. 2.0% [2/101], $P < 0.001$).

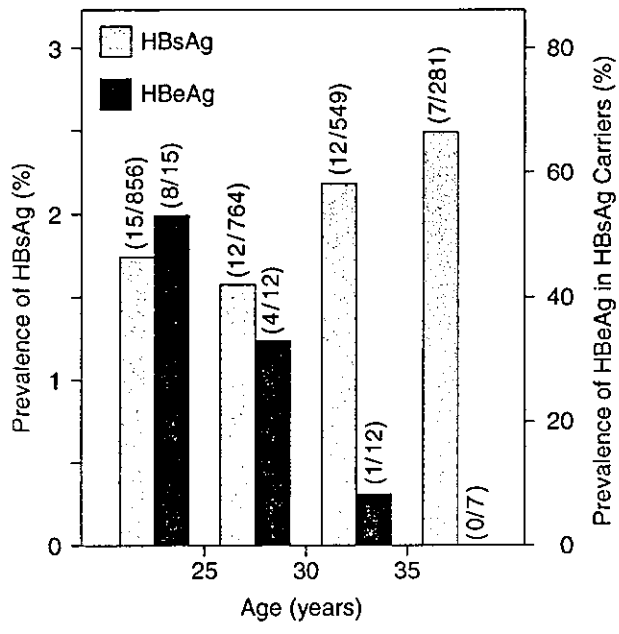


Fig. 2. Age-specific prevalence rates of HBsAg and HBeAg in 2,450 pregnant women in Bali.

## DISCUSSION

In surveys for serological markers of HBV and HCV infections among blood donors performed in 1991 in Jakarta, Indonesia, HBsAg was detected in 5.8% and anti-HCV in 17.7% [Sastrosoewignjo et al., 1991]. HBV and HCV strains indigenous to Indonesia are reported in blood donors and hepatitis patients there [Sastrosoewignjo et al., 1991; Hadiwandowo et al., 1994; Mulyanto et al., 1997]. Data are still inadequate, however, on serological markers of HBV and HCV infections, as well as HIV infection, in the general population in Bali that is isolated from the other Indonesian archipelagos by the sea. Nor are there any data available for the exposure to HEV in Bali, except for a recent report by Wibawa et al. [2004] on 276 family members of chronic liver disease and 797 voluntary blood donors.

Taking advantage of the routine screening for HBsAg, 2,450 pregnant women in Bali were tested for serological markers of HBV, HCV, and HEV infections, and HIV infection. The prevalence of HBsAg examined during 5 months in 2003 was significantly lower than that in 1993 (1.9% vs. 2.6%, $P < 0.045$). It is not certain, however, how the prevalence of HBV markers surveyed

TABLE II. Frequencies of Anti-HEV in Hindu and Non-Hindu Women in Various Districts of Bali

| Districts | Hindu | Non-Hindu | Differences |
|---|---|---|---|
| Bangli | 25/92 (27%) | 0/1 | NS[a] |
| Denpasar | 33/175 (19%) | 2/21 (9.5%) | NS |
| Gianyar | 32/100 (32%) | 0/1 | NS |
| Karangasem | 6/39 (15%) | 0/2 | NS |
| Klungkung | 19/91 (21%) | 0/7 | NS |
| Negara | 11/90 (12%) | 0/10 | NS |
| Singaraja | 19/99 (19%) | 0/5 | NS |
| Tabanan | 4/83 (4.8%) | 0/3 | NS |
| Total | 149/769 (19%) | 2/50 (4.0%) | $P < 0.012$ |

[a]Not significant.

in pregnant women who give birth to their babies in hospitals is extended to the general population in Bali, where the majority of deliveries are conducted by midwives at home. Wibawa et al. [2004] detected HBsAg in 38 of the 797 (5%) voluntary blood donors and 18 of the 276 (7%) family members of patients with chronic liver disease from Bali. Their results stand at a substantial variance with ours.

Anti-HCV in pregnant women in Bali was low at 0.04%, in contrast to the detection of anti-HCV in 17.7% of voluntary blood donors in Jakarta [Sastrosoewignjo et al., 1991]. Although data are lacking for the prevalence of anti-HCV in the Balinese, it is reasonably expected to be low in the general population of Bali; Wibawa et al. [2004] detected anti-HCV in 6 of the 796 (0.8%) blood donors. With rapid increases of immigrant and tourists into Bali, however, the exposure to HCV may expand in the foreseeable future. In support of this view, the prevalence of anti-HIV among female sex workers in Bali is reported to be higher for immigrants than the Balinese [Ford et al., 2000].

To address possible concerns on the sensitivity of locally produced assays for HBsAg and anti-HCV, the Entebe kits for these viral markers have been used during the past 18 and 8 years since 1986 and 1996 for HBsAg and anti-HCV, respectively, for screening blood units at many blood centers in Lombok and other islands of Indonesia. Indisputable decrease (to practically zero) in the incidence of posttransfusion hepatitis B and C since then would indicate a high sensitivity of these tests.

HIV infection has become very rare in female sex workers in Bali (0.2%), although the frequencies of sexually transmitted disease such as gonorrhoea (60.5%), chlamydia (41.3%), and human papilloma virus (37.7%) remain very high [Ford et al., 2000]. The reasons for such a low exposure to HIV in the Balinese, even in high-risk groups, are not clear. It is a surprise, especially because a pandemic of HIV is expected in Indonesia [Anonymous, 1996]. Isolation from the other areas of Indonesia, surrounded by sea, may have prevented exposure to HIV and HCV that have been introduced more recently than HBV. In addition, heavy punishments imposed on the use of illegal drugs may have prevented the spread of these blood-borne viruses there.

Overall, anti-HEV was detected in 18% of pregnant women living in eight jurisdiction, at a frequency comparable to 18%–20% recently reported in Bali [Wibawa et al., 2004]. Previous findings point to the zoonotic food-borne transmission that may play an important role in HEV infection among Japanese people. For instance, some individuals who ate sashimi prepared from deer caught in the wild [Tei et al., 2003] or feral boar's liver in the raw [Matsuda et al., 2003] developed acute or fulminant hepatitis E. In addition, Yazaki et al. [2003] have suggested the ingestion of pig's liver as a major risk factor for hepatitis E among residents of Hokkaido, Japan. These observations in Japan instigated us to look into whether zoonotic food-borne transmission of HEV also occurs in inhabitants of Bali where anti-HEV has not been surveyed extensively. As the results, the prevalence of anti-HEV was found to be more frequent in Hindu than Muslim residents of Bali. Muslims are strictly prohibited from eating or touching pigs, while Hindus have no such restrictions.

When the prevalence of IgG anti-HEV was compared among pregnant women in eight districts of Bali, significant differences were found among them in a range from 4.7% (4/86) in Tabanan to 32% (32/101) in Gianyar. An even more striking difference was noted in pregnant women between Hindus and non-Hindus (mostly Muslims and a few Christians) (19% [149/769] vs. 4.0% [2/50], $P < 0.012$).

Since the religion of Bali is predominantly Hindu, a random sampling of the Balinese would hardly reflect the anti-HEV status in non-Hindus, as in the study of Wibawa et al. [2004] and ours. Furthermore, the exposure to HEV may be influenced by sanitary conditions and water quality that differ in various areas of Bali. These factors taken into considerations, pregnant women living in a restricted area of Denpasar City (Sanglah) were examined for evaluating the influence of religion on HEV exposure. As the results, anti-HEV was significantly more frequent in Hindus than Muslims (20% [20/102] vs. 2.0% [2/101], $P < 0.001$).

The observed differences in the prevalence of anti-HEV would be attributed to distinct life-styles of the Balinese in association with their religions. Among many differences dependent on religions, those in the dietary habit are prominent. Hindu families in Bali typically keep pigs within the household, as a source of food, and often eat grilled pork that can be undercooked. In contrast, Muslims are rigorously prohibited from tasting or even touching pigs by their religion. Thus, it would be reasonable to implicate close contacts with pigs, along with the ingestion of domestic pork, in a high exposure to HEV among Hindus living in Bali. Although "water-borne" transmission of HEV has been reported in Indonesia [Corwin et al., 1997], the results obtained in this study suggest an alternative mode of HEV transmission in Bali that is "pig-borne." In actuality, pigs in Bali are highly contaminated with HEV; anti-HEV is detected in more than 70% of them [Wibawa et al., 2004]. Furthermore, zoonotic infections are common among children and teenagers in Bali [Chomel et al., 1993].

Women in Bali appear to have been exposed to HEV long before the pregnancy. The absorbancy for anti-HEV in ELISA was mostly low and HEV RNA was not detectable in any of 20 sera with a high absorbancy (>1.50). Hence, the risk of developing fulminant hepatitis by HEV infection during the pregnancy would be lower in Bali than in India [Kar et al., 1997].

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