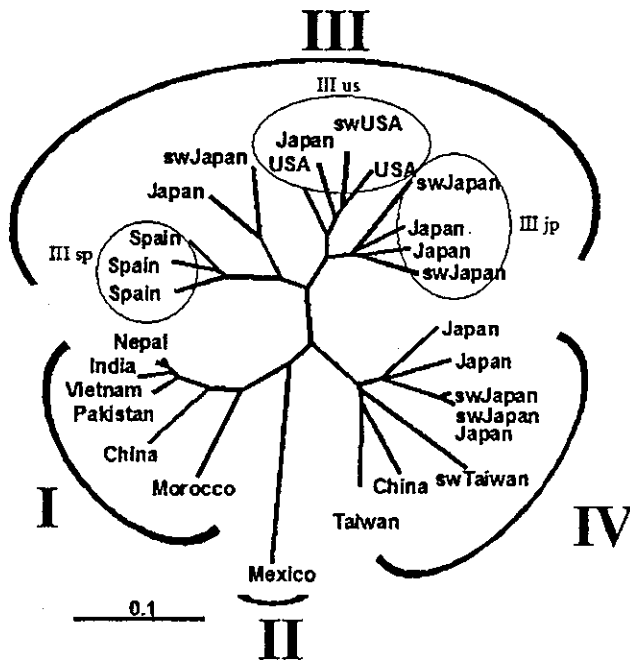
表1 E型肝炎ウイルスの遺伝子型とその分布

E型肝炎ウイルス遺伝子型	保有動物	ウイルス分離された主な地域
I型	ヒト	アジア（インド、ネパール含む)、アフリカ (E型肝炎多発地域)
	ドブネズミ	ネパール
II型	ヒト	メキシコ、アフリカ (E型肝炎多発地域)
III型	ヒト	日本、韓国、ヨーロッパ、北米
	ブタ	日本*、台湾、韓国、ヨーロッパ、北米
IV型	ヒト	日本、台湾、中国、ベトナム
	ブタ	日本*、台湾、中国、インド、インドネシア

*日本では、豚以外にイノシシからIII型、IV型 HEV が、シカからIV型 HEV が検出されている。

図1 E型肝炎ウイルスの遺伝子系統樹



E型肝炎ウイルスのORF2遺伝子領域について調べた分子系統樹。遺伝子型がI～IV型の4種に分けられ、それぞれ分離された地名が記されている。地名の前の「sw」はブタ(swine)由来であることを意味する。その他はヒト由来である。

混じっているのが分かる。さらに、日本、台湾などの国ではヒトとブタから分離されたHEVが国ごとに非常に近縁である例が示されている。これは、各地方でヒト・ブタ間のHEV感染があることを疑わせる傍証の一つになっている。実際、日本で検出されたヒト由来HEVとブタ由来HEVが極めて似ている例（核酸配列で98%以上一致）が複数報告されている。

実験感染を行って、ブタ由来III型HEVは赤毛ザルに感染し急性肝炎を発症させ、逆にヒト由来I型HEVはブタに感染し、ブタへの同居感染も起こったが両者とも無症状であることが確認されている[1]。これはヒトとブタの間でHEVが伝播しうることを強く示唆したデータである。

2．わが国での動物由来E型肝炎ウイルスによるヒト急性E型肝炎の例

わが国のE型肝炎患者から検出されたHEV遺伝子を解析すると、外国のE型肝炎多発地から帰国した患者からはI型HEVが分離され、国内感染と考えられるケースではIII型かIV型のHEVが分離されることが多いという。しかも、国内感染例の幾つかは動物由来HEV感染症、つまり人獣共通感染症、と考えられている。もちろん、その他感染ルートの不明な国内感染例も多い。

2003年になってわが国から、動物に由来するHEV感染でヒトが急性E型肝炎を発症したケースが4件報告された。

1．野生シカ肉の刺身を食べた4人が急性肝炎。シカ肉 HEV と患者 HEV の遺伝子配列一致。(兵庫県)(東芝病院報告　8月)(2)
2．イノシシ内臓肉を生食した2人が急性劇症肝炎。一人死亡。(鳥取県)(東芝病院報告　9月)(3)
3．E型急性肝炎患者10人中9人が2－8週前にブタレバーを生食していた。肉屋で売られているブタレバーパックの7/363(1.9%)に HEV 遺伝子検出。(北海道)(自治医大報告　9月)(4)
4．イノシシ肉を食べた12人中8人が HEV 感染、5人発症。(長崎医療センター報告　11月)

世界のE型肝炎多発地では、大流行の感染経路が主に HEV に汚染した井戸水、河川の水、洪水後の水、など生水を介した感染とされるのに比べ、これら日本の感染例はシカ、イノシシ、ブタなどの食肉を介した感染であるのが特徴である。HEV の場合、ヒトからヒトへの伝播は多発地域でも非多発地域でも殆どないとされている。

3．ヒトでの抗 HEV 抗体保有率

E型肝炎流行地域での抗 HEV IgG 抗体保有率は80%以上あるのに比べ、非流行地域と考えられている日本では、平均5%程度と報告されている。ところが、日本でのさらに詳細な調査によると地域間に抗体保有率の差が見られるものの、抗体保有率は20代までは非常に低いが、加齢と共に上昇し、40代、50代では20%から30%にのぼる県もあった(5)。この傾向は日本だけではなく、欧米を含む他の非流行地域でもE型肝炎発症率の低さに比較して、抗体保有率が高く、多くの不顕性感染が存在していると考えられている。

では、この非流行地域での不顕性感染の感染源はどこにあるのだろうか？もし畜産物を介して感染しているとしたら、なぜ成人にならないと抗体保有率が高くならないのか、など多くの疑問が残されている。

4．日本の養豚場でのE型肝炎ウイルス

自治医大の岡本宏明教授らが全国の養豚場のE型肝炎ウイルスの保有状況調査を行った(6)。その調査報告の要点は次の通りである。

1)　25農場（北海道、青森、秋田、宮崎、鹿児島）について1農場当たり2－6ヶ月齢の豚100頭、計2,500頭を調査した。
2)　血中抗体陽性率はすべての農場で6ヶ月齢の豚で80－100%に達する。
3)　血中 HEV 遺伝子検出率は3－4ヶ月齢で平均13－15%。
4)　遺伝子型Ⅲ とⅣが検出される。
5)　同一農場内の HEV 遺伝子は似ている。
6)　日本人から分離された HEV に非常に近いものも分離される。

わが国の養豚における高抗体保有率に関しては、動物衛生研究所の木嶋眞人・山本孝史・恒光裕らが感染研の武田直和・李天成博士から分与された組換え HEV ORF2 蛋白抗原を用いて ELISA 法による抗体調査を行い、自治医大が示した養豚の高抗体保有率を再確認している。世界各国から養豚の抗 HEV 抗体保有率が報告されているが、用いる抗原によって検出感度に違いがあると考えられるものの、欧米も日本と同様に抗体保有率は高いものと推測される。

5．養豚従事者での抗 HEV 抗体保有率

前述のように、E型肝炎非多発国であっても健常人が抗 HEV 抗体をある程度持っている。養豚に接触する機会の多い養豚従事者についても、我々は知っておく必要がある。今のところ日本での調査報告はないが、外国での調査報告があり、いずれも統計的な有意差を持って養豚関係者の方が抗体保有率が高いとしている。

台湾では、抗体保有率が対照者4%（n＝50）に対し、養豚従事者27%（n＝30)、食肉加工業者15%（n＝20）であった(7)。東欧旧ソ連邦のモルドバでは、対照者25%（n＝255）に対し、養豚家は51%（n＝264）だった(8)。米国に関しては2報あり、一報では(9)、幾つかの州の献血者17－18%（n＝400）に対し、同州のブタ専門獣医23－26%（n＝295)、もう一報では(10)、非養豚従事者2%（n＝127）に対して、養豚従事者11%（n＝165）であった。

詳細なアンケート調査を行った Meng ら(9)の報告で、抗体陽性率と関係ある因子を探したが特定できなかったとしている。すなわち、「注射針で刺したり傷を作った経験」「ブタと接触する時間」「職種（研究、臨床、学生、民間会社獣医)」は抗体陽性率に関与するとは言えなかった。また、獣医、献血者（対照群）とも加齢とともに抗体保有率が高くなる傾向は同様であった。従って、養豚業者の高抗体陽性率の理由はまだ不明である。

これらの養豚従事者の抗体保有率が高いという報告に対し、肝炎発症が高いという報告は見あたらない。感染する機会は一般の人より高いが、多くは不顕性感

染に終わっていると解釈すべきであろうか。

6．おわりに

1997年、ヒトから分離される HEV と非常に似たウイルスがブタから分離されたことに端を発して、E型肝炎非流行地である日本や欧米でも、ブタやイノシシにはヒトに感染しうる HEV が高頻度に存在している実態が明らかになった。さらに、この1－2年でブタの HEV がヒトへ感染し急性肝炎を起こしたのではと疑われる症例が少数ではあるが明らかにされた。それらの「事故例」は今のところ日本だけで報告されている。やはり、ブタの肝臓ないし肉を生あるいは不十分な加熱で食べるという日本人の食嗜好と無関係ではなかろう。現在までの調査では、日本の養豚農場のほとんどは HEV を持っているであろうと考えられるが、糞口感染で容易に同居豚に水平感染するらしく、ウイルスを農場から取り除くのは簡単ではない。現状では豚レバー、豚肉を十分に加熱して食べるのが最善の策と思われる。

参考文献

1．X. J. Meng et al., J Virol 72, 9714 (1998).
2．S. Tei, N. Kitajima, K. Takahashi, S. Mishiro, Lancet 362, 371 (Aug 2, 2003).
3．H. Matsuda, K. Okada, K. Takahashi, S. Mishiro, J Infect Dis 188, 944 (Sep 15, 2003).
4．Y. Yazaki et al., J Gen Virol 84, 2351 (Sep, 2003).
5．F. Li et al., J Med Virol 60, 379 (2000).
6．M. Takahashi et al., J Gen Virol 84, 851 (Apr, 2003).
7．S. Y. Hsieh et al., J Clin Microbiol 37, 3828 (1999).
8．J. Drobeniuc et al., J Infect Dis 184, 1594 (Dec 15, 2001).
9．X. J. Meng et al., J Clin Microbiol 40, 117 (2002).
10．M. R. Withers et al., Am J Trop Med Hyg 66, 384 (Apr, 2002).

日本語参考ウエッブサイト

1) http://idsc.nih.go.jp/idwr/kansen/k04/k04_13/k04_13.html
国立感染症研究所 HP／感染症情報センター／感染症の話／E型肝炎　武田直和
2) http://niah.naro.affrc.go.jp/disease/HEV/links.html
動物衛生研究所 HP／疾病情報／Hepatitis E Virus について

＜短　報＞

野生イノシシの肝臓，血液からＥ型肝炎ウイルス遺伝子の検出

三好　龍也[1)]　李　天成[2)]　武田　直和[2)]　宮村　達男[2)]　田中　智之[1)]

目的：Ｅ型肝炎は主に東南アジア，インド，中国などの国々で散発的に，時には水系感染による大流行の発生がみられている疾患である．日本でのＥ型肝炎は，これらの流行地域からの輸入感染症として考えられてきたが，最近，海外渡航歴のないＥ型肝炎患者の発生や，野生イノシシ[1)]や野生シカ[2)]肉の生食による国内での感染事例が報告されている．また，ブタ[3)]，野生ドブネズミ[4)]などからＥ型肝炎ウイルス(HEV)の遺伝子が検出されており，Ｅ型肝炎は人獣共通感染症として注目されてきた．

そこで我々は，自然界におけるHEVの実態調査のため，野生のイノシシとシカを対象としてHEV保有調査を行った．

材料：2003年11月の狩猟解禁から2004年1月にかけて，紀伊半島の和泉山脈から南方の紀南地区で狩猟されたイノシシ9頭と，シカ2頭の血液と肝臓(シカ1頭については血液のみ)を用いた．抗HEV IgG抗体の測定はイノシシ血清についてのみ行った．HEV遺伝子の検出は，イノシシ，シカのすべての検体について行った．

方法：1. HEV抗体測定；抗HEV IgG抗体の測定は，李ら[5)]の方法に準じた．すなわち，組換えバキュロウイルスから作製されたHEVウイルス様粒子を固相抗原としたELISA法を用いた．検体血清は1：200希釈して用い，イノシシ血清に対する二次抗体にはHRP-conjugated rabbit anti-swine IgG(ICN Cat #55826)を用いた．

2. HEV遺伝子検出法；落合[6)]らの方法に準じ，肝臓検体については20%肝組織乳剤を10,000 xg，20分間遠心した上清を，また，血液検体については，3,000 xg，15分間遠心した血清をそれぞれRNA抽出用検体とした．RNA抽出は，ISOGEN-LS(ニッポンジーン社製)を用い，nested RT-PCRによりHEV遺伝子の検出を行った．得られたPCR産物はABI PRISM 310 Genetic Analyzerを用いてダイレクトシーケンスを行い，塩基配列を決定した．

結果：1. HEV抗体測定；9頭のイノシシについて血清中の抗HEV IgG抗体を検査した結果，2004年1月29日に和泉山脈で捕獲された1頭が，約400倍の抗体価を有していた．他の8頭は200希釈血清のOD値が，全て0.15以下で陰性と判断した．

2. HEV遺伝子検出；イノシシ9頭の内，上述のIgG抗体陽性の1頭の血液と肝臓からHEV遺伝子が検出された(図1)．これらのHEV遺伝子の塩基配列は同一であった．系統樹解析の結果，得られた塩基配列は国内のＥ型肝炎患者由来の塩基配列AB082567と約90%の相同性を示し，遺伝子型III型に分類された(図2：Sakai 9)．その他のイノシシの検体およびシカ2頭からHEV遺伝子は検出されなかった．

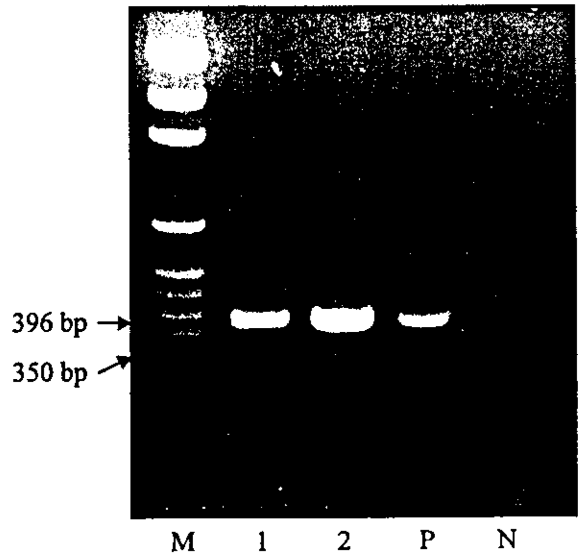


図1　野生イノシシからのHEV nested PCRの結果：378 bpの部位にPCR産物が認められる．(M；Marker，1；血液，2；肝臓，P；positive control，N；ngative control)

考察：HEVは細胞培養によるウイルス分離が確立されていない病原ウイルスの一つである．従って，ウイルス学的診断はHEV抗体測定とHEV遺伝子検出が，もっとも科学的根拠に基づいた診断法と考えられている．今回用いたこれらの測定方法は，すでに確立されたものである．

我々が調査した野生イノシシ9頭中，1頭から抗HEV IgG抗体およびHEV遺伝子が検出された．昨年度の厚生労働科学特別研究事業「食品に由来するＥ型肝炎ウイルスのリスク評価に関する研究」班会議においても，愛知県で捕獲された野生イノシシからHEV IV型遺伝子の検出報告があった．今回の成績は，一つには，本邦における野生イノシシからのHEV III型遺伝子の最初の検出例と考え，また二つには，本調査研究によって国内の野生イノシシにはHEVが浸淫している可能性が強く推測された．しかし，今回調査したシカについては，HEV遺伝子は検出されなかった．シカ肉によるHEV感染事例を考えると，今後も調査対象動物から除外できず，広範な検査が必要である．

Ｅ型肝炎が人獣共通感染症として注目されている中，HEVの自然界での宿主動物の特定および感染実態の調査は，HEVのヒト-動物における感染経路の解明，予防対策

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＜受付日2004年5月31日＞

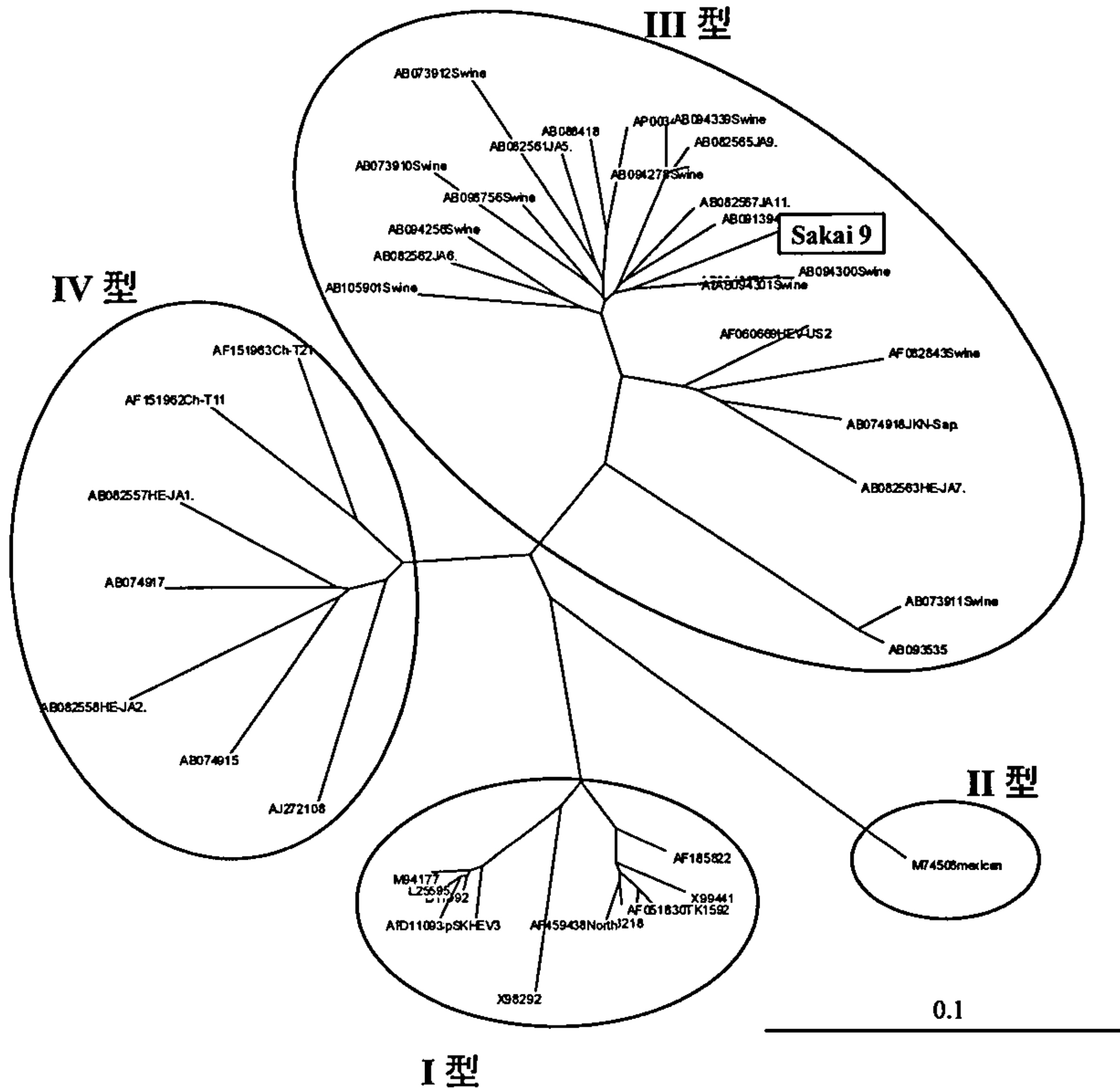

図2　今回検出したSakai 9 HEV系統樹解析（NJ法）：III型遺伝子グループに分類される．

を考える上で重要である．今回の成績に加えて，今後，HEVの広範囲な実態調査が必要と考えられる．さらに，このHEVが日本土着のものなのか，あるいは輸入されたものなのかなどの感染症学的解析のみならず，食の安全という社会衛生学的な観点からも，ヒトにおける疫学調査やHEV遺伝子解析は今後も重要な研究課題と考える．

謝辞：本研究の一部は平成15年度厚生労働科学特別研究事業「食品に由来するE型肝炎ウイルスのリスク評価に関する研究」班（宮村達男主任研究者）の研究補助金によった．

索引用語：E型肝炎，野生イノシシ，HEV遺伝子

文献：1）Matsuda H, Okada K, Takahashi K, et al：J Infect Dis 188(6)：944, 2003　2）Tei S, Kitajima N, Takahashi K, et al：Lancet 362：371—373, 2003　3）Yazaki Y, Mizuo H, Takahashi M, et al：J Gen Virol 84：2351—2357, 2003　4）He J, Innis L B, Shrestha P M, et al：J Clin Microbiol 40：4493—4498, 2002　5）Li T C, Zhang J, Ishibashi M, et al：J Med Virol 62：327—333, 2000　6）「E型肝炎検査マニュアル」衛生微生物技術協議会レファレンス委員会編（発行予定）

＜速　報＞

本邦に棲息する野生猪の HEV 感染に関する実態予備調査

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緒言：本邦のＥ型肝炎ウイルス（HEV）感染においては zoonosis の重要性がつとに指摘されている[1～3)]．我々は本邦において最近頓にその棲息数が増加しつつある野生猪（*Sus scrofa leucomystax*）について，もしそれが HEV の reservoir であるとすれば看過すべからざる感染源になり得るとの観点から，HEV 感染状況の予備的調査を実施した．

方法：兵庫県在住のハンターの協力を得て，2004 年 3 月末から 4 月初頭迄の期間に兵庫県中部の多可郡及び西脇市において計 7 頭の猪を捕獲し，肝臓及び血液をサンプリングし，HEV RNA と HEV 抗体の検出を試みた．前者の検出は既報[4)]に従い，後者（IgG）の検出は市販予定キット（デンカ生研株式会社）の二次抗体を抗ブタ IgG 抗体で置換する方法により行った．

成績：7 頭の猪のうち 4 頭に IgG class anti-HEV ELISA OD＞1.0 を認めた．そのうち特に抗体価の高かった 3 頭（ELISA OD＞2.0）においては，全例，肝臓及び血液中の HEV RNA が陽性であった．この 3 頭はいずれも若く（推定年齢 1 歳未満），HEV RNA 陰性であった他の 4 頭に較べて，明らかに低体重であった（表）．

考案：猪の HEV 抗体測定系は未だ標準化されていない故，確定的なことは言えないが，もし本調査で用いた in-house ELISA の OD＝1.0 をカットオフとすれば，本調査における兵庫県棲息野生猪の HEV 抗体保有率は 4/7（57%）であり，一方 HEV RNA 保有率は 3/7（43%）であり，いずれも驚嘆すべき高値を示した．HEV RNA 陽性を示した猪が若年齢であったことは，我が国の飼育豚で見られた所見[5)]と同一である．これらの所見は，飼育豚におけると同様に，兵庫県中部に棲息する野生猪集団においては HEV 感染が endemic であることを強く示唆している．隣県の鳥取から猪ナマギモ摂取後に発症した重症Ｅ型肝炎が 2 例報告されたことや[2)]，長崎からの猪バーベキュー後Ｅ型肝炎集団発生の報告にも鑑みて[3)]，我が国におけるヒトＥ型肝炎の重要な感染源の一つとして猪（特に若い猪＝体の縞模様から「ウリ坊」と呼ばれる）を考慮せねばならない．

Table　HEV markers in the 7 boars

Age (years)	Body weight (kg)	Anti-HEV (ELISA OD)	HEV RNA
＜1	6	＞2.000	＋
＜1	7	＞2.000	＋
＜1	7	＞2.000	＋
1	25	0.322	−
1	25	0.302	−
1	25	0.238	−
5	45	1.327	−

謝辞　本調査に御協力頂いたハンターの藤本秀道氏と兵庫県森林林業課長北村富士雄氏に感謝する．

索引用語：Ｅ型肝炎，zoonosis，猪

文献：1) Tei S, Kitajima S, Takahashi K, et al. Lancet 2003；362：371―3　2) Matsuda H, Okada K, Takahashi K, et al. J Infect Dis 2003；188：944　3) Tamada Y, Yano K, Yatsuhashi H, et al. J Hepatol 2004；40：869―70　4) Takahashi K, Kang JH, Ohnishi S, et al. Intervirology 2003；46：308―18　5) Takahashi M, Nishizawa T, Miyajima H, et al. J Gen Virol 2003；84：851―62

英文要旨

HEV infection in wild boars in Japan

Kitajima N[1)], Takahashi K[2)], Abe N[2)], Mishiro S[2)*]

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Hepatitis E virus (HEV) transmission via zoonosis has been implicated in sporadic cases of hepatitis E in Japan. We undertook a preliminary survey to see if wild hoar might be an HEV reservoir. Of 7 boars hunted at Taka-gun and Nishiwaki-city, Hyogo, Japan, from late March to early April 2004. 4 (57%) were positive for IgG class anti-HEV, among which 3 (43%) were positive for HEV RNA as well. These RNA-positive 3 boars were younger than the other 4 RNA-negative ones, based on the body weight at capture. These results stuggest that HEV is endemic in wild boars in this region, and may serve as an infection source for human beings and other animals.

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To the Editor

HEV infection in wild boarについて-1

編集長殿，雑誌『肝臓』9月号掲載の三好龍也氏等の短報論文，「野生イノシシの肝臓，血液からE型肝炎ウイルス遺伝子の検出」（肝臓 2004；45：509—10）を非常に興味深く読ませていただきました．

しかし，一つ残念なことには，その論文で報告されているHEV　Sakai 9という分離株の塩基配列のDDBJ/EMBL/GenBank accession numberが記載されていません．大変重要な情報ですから，是非それを記載していただきたかったと思います．できれば，それを著者から御教示いただいて，このTo the Editor欄にでも公開していただきたいと思います．よろしくご検討下さい．

2004年10月4日

市立加西病院内科
北嶋　直人

一拝復，北嶋直人先生．先生の御指摘に全く賛同致します．弁解めきますが，編集委員長である私でさえ三好龍也先生等の当該論文を9月号発行（何故か配本が10月にずれこみました）後に初めて読みました．その理由は，新編集委員会が仕事を引き継ぐ前に，既にその論文は査読・編集等のプロセスをあらかた終了していたからだろうと思います．もし私が査読責任者であったなら，必ず著者にaccession no.の記載を要求していたと思います．それは此の種のリポートに於いて殊更重要な情報だからです．

そこで，先生の御要望に応えるべく，同論文の共著者である国立感染研の武田直和博士に問いあわせ，現在返事待ちの状態です．入稿日までに間に合えばここにHEV Sakai 9のaccession no.を記載しますが，そうでない場合には11月号で公開します．

2004年10月4日

「肝臓」編集委員長・三代　俊治

HEV infection in wild boarについて-2

雑誌肝臓編集委員会御中，9月号の短報「野生イノシシの肝臓，血液からE型肝炎ウイルス遺伝子の検出」[1]を拝読しました．著者らは野生イノシシ9頭中1頭に血液中抗体価の高い個体を見出し，さらにそのイノシシの血液，肝臓中から遺伝子型III型のHEV-RNAの検出に成功しております．

2003年，HEVと食肉の関連が社会問題にまで発展しましたが，その食肉とは，シカ，ブタ，そしてイノシシでした．地区によっては，市販のブタ肝臓からHEVが一定の割合で検出され食肉業界には衝撃的でした[2]が，多数例を検討した高橋ら[3]の研究成果からは3—4カ月齢で9割ものブタがHEVに感染するが，出荷時（6カ月齢）のウイルス検出率は非常に低いと考えられています．シカについては三代ら[4]の報告によりますと，肝臓中に1/100頭の割合で検出されたとありますのでさほど高い割合ではありません．

一方，イノシシは遺伝学的にブタと非常に近縁であり，ブタと同様，幼若期に一過性感染をきたすと考えても不思議でありません．私たちも九州の一地域でイノシシ肉に起因すると考えられる集団感染事例を報告し[5]，その追跡調査の一環として付近の野生イノシシ肉（筋肉）を検討しましたが，preliminaryな結果ながら25頭中少なくとも3頭にHEV-RNAを検出しております．以上のことから，野生イノシシは一定の割合（おそらく幼若期に）でHEV-RNAを保有しており，狩猟によって年（月）齢にかかわらず食されることから，HEV感染のリスクとしては上記動物の中で最も高いと推察されます．

今回の三好らの報告を足がかりに，野生イノシシの幅広い調査を早急に行うことが望まれます．

References: (1)三好達也，李天成，武田直和等．肝臓2004；45：509－10. (2) Yazaki Y, Mizuo H, Takahashi M, et al. J Gen Virol 2003；84：2351－7. (3) Takahashi M, Nishizawa T, Miyajima H, et al. J Gen Virol 2003；84：851－62. (4)三代俊治．本邦におけるE型肝炎の診断・予防・疫学に関する研究班平成15年度報告書．(5) Tamada Y, Yano K, Yatsuhashi H, et al. J Hepatol 2004；40：869－70.

2004年10月6日

独立行政法人国立病院機構長崎医療センター
矢野　公士

— 拝復，矢野公士先生．恰も Editorial であるかの如き優れて enlightening な解説を御投書頂き有難うございました．HEV 感染の野生動物 reservoirs の中でイノシシは最も重要な位置を占めると私も考えています．しかもイノシシは日本国内の此方彼方で近年急増しつつあります．とき恰も食欲の秋，読者諸兄姉，gibier（地ビールに非ず，ジビエ［仏語］）を楽しむ際にはイノシシを生食せられぬよう御注意あれ！

2004 年 10 月 6 日

「肝臓」編集委員長・三代　俊治

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Rapid Communication

Complete or near-complete nucleotide sequences of hepatitis E virus genome recovered from a wild boar, a deer, and four patients who ate the deer☆

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Abstract

Zoonosis has been implicated in hepatitis E virus (HEV) transmission. We examined wild boar living in a forest of Hyogo prefecture, Japan, and found HEV RNA in three of seven boars. A full-genome HEV isolate from one of them was revealed to be 99.7% identical to a previous isolate from a wild deer hunted in the same forest and to those from four patients who contracted hepatitis E after eating raw meat of the deer. These findings suggest an interspecies HEV transmission between boar and deer in their wild life, and that both animals might serve as an infection source for human beings as suggested previously.
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Keywords: Hepatitis E virus (HEV); Single-stranded RNA virus; Zoonosis; Interspecies transmission

Introduction

We reported previously a case of zoonotic food-borne transmission of hepatitis E virus (HEV) from a wild deer (*Sika* deer, *Cervus nippon nippon*) to human beings, in which four patients developed hepatitis E after eating *Sashimi* of the deer meat (Tei et al., 2003). We also reported a case where consumption of the raw liver (*Namagimo*) of wild boar might have been the cause of severe hepatitis E in two patients (Matsuda et al., 2003). Tamada et al. (2004) also reported an outbreak of HEV infection in those who had participated in a boar-barbecue party. These findings prompted us to investigate the wild boar living in Japan (*Inoshishi*, *Sus scrofa leucomystax*) as a possible reservoir of HEV. A very preliminary study with only seven boars hunted March and April 2004 in Hyogo prefecture, Japan, indicated an unexpectedly high prevalence of HEV infection among them: four were positive for antibodies to HEV (anti-HEV) and three of them were positive for HEV RNA as well (Kitajima et al., in press). In the present report, we describe our results of genetic analyses of an HEV isolate (JBOAR1-Hyo04) recovered from one of the three HEV RNA-positive boars. And also, we obtained near-full-genome sequence from the deer-derived HEV, for which we had reported previously only a 326-nt sequence (Tei et al., 2003). To our knowledge, there have been no reports describing full or near-full-genome HEV sequences that were recovered from wild boar or deer.

Results

The HEV isolate named "JBOAR1-Hyo04" was obtained from a wild boar hunted April 8, 2004, in the same forest of Taka-gun, Hyogo prefecture, Japan (Fig. 1), where the HEV-

☆ The nucleotide sequences reported in this paper will appear in DDBJ/EMBL/GenBank databases under accession numbers AB189070–AB189075.

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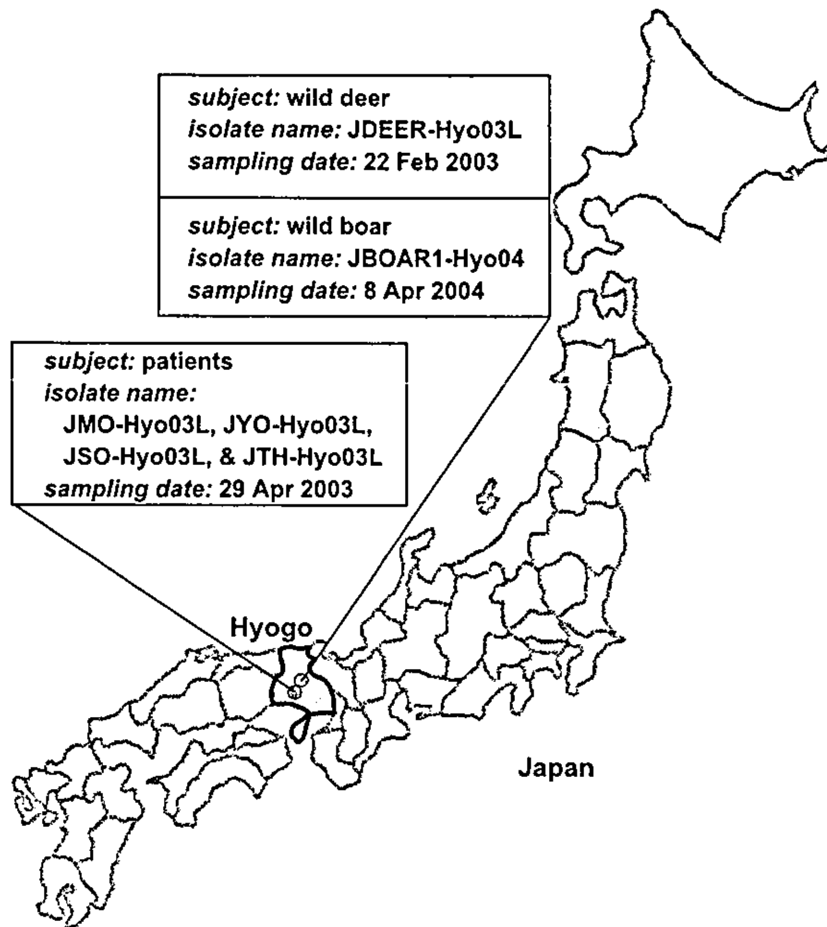

Fig. 1. The HEV isolates analyzed in this study and the locations wherefrom they were derived.

infected deer we reported previously (Tei et al., 2003) had been hunted a year before (February 22, 2003). HEV RNA, detectable in both the liver and serum from this boar, was subjected to amplification and sequencing by RT-PCR for its entire genome. The nucleotide sequence of JBOAR1-Hyo04 thus obtained was 7247 nt in length, comprising of 5'UTR (nt 1–26), ORF1 (nt 27–5138 for 1703 aa), ORF2 (nt 5173–7155 for 660 aa), ORF3 (nt 5135–5503 for 122 aa), 3'UTR (nt 7156–7226), and poly-A tail (nt 7227–7247).

The JBOAR1-Hyo04 isolate co-clustered in a phylogenetic tree (Fig. 2) with the HEV isolate from the above-mentioned deer (JDEER-Hyo03) as well as those from four patients who developed hepatitis E after eating the deer meat (JSO-Hyo03, JTH-Hyo03, JYO-Hyo03, and JMO-Hyo03), whose nucleotide sequences were known for only a 326-nt part of ORF1 (DDBL/EMBL/GenBank accession numbers AB114179–AB114183). Then, we tried to determine much longer sequence of the deer and patients-derived HEV genome for extended comparison with the boar-derived HEV, and obtained a 7230-nt sequence for the deer (JDEER-Hyo03L) and 7180-nt sequences for the patients (JSO-Hyo03L, JTH-Hyo03L, JYO-Hyo03L, and JMO-Hyo03L). Although these sequences did not cover the complete genome, they did cover entire coding regions (ORF1 + ORF2 + ORF3) at least.

Nucleotide identity between JBOAR1-Hyo04 and the deer/human isolates compared for the length of about 7.2 kb was 99.7%, while that between the isolate from deer and those from the patients was 99.9%. Comparison with complete or near-complete HEV isolates so far reported to date showed that these six isolates segregated to a mini-cluster completely independent from other isolates in the genotype III (Fig. 3). In addition, they shared almost 100% identical sequence even at the hypervariable region (HVR) within ORF1.

These findings and the fact that the deer and the boar were hunted in the same forest suggested that an interspecies transmission of HEV had been occurring between these animals in the shared habitat.

Discussion

Firstly, we believe that this is the first report to describe full or near full-genome HEV sequence recovered from wild boar and deer.

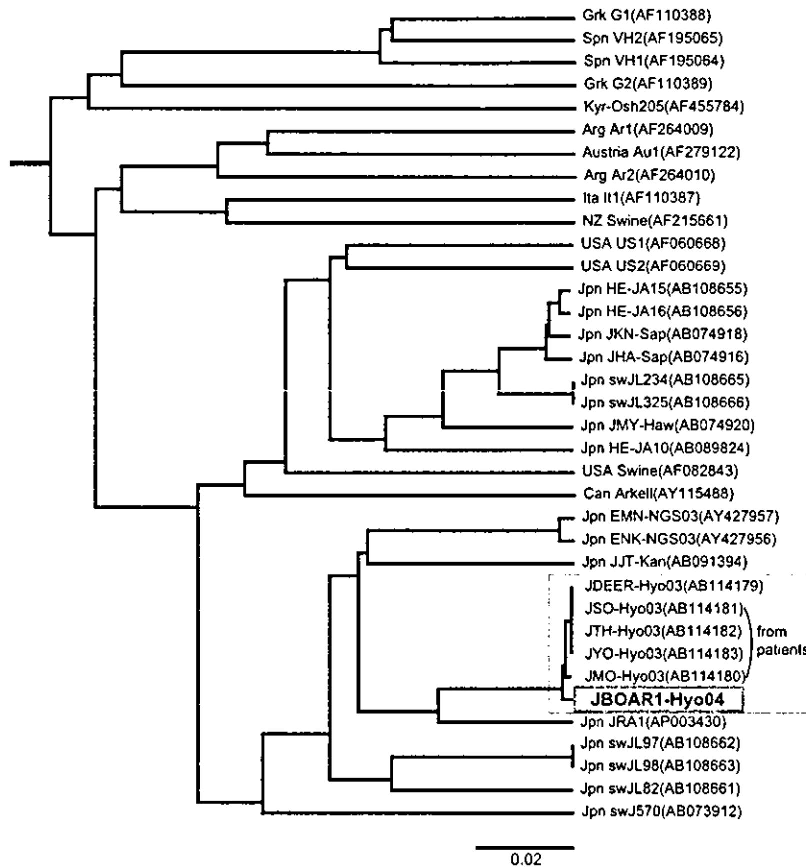

Fig. 2. Phylogenetic tree (UPGMA) based on a 326-nt partial sequence of ORF1. The sequence of JBOAR1-Hyo04 was compared to those so far reported as genotype III isolates at a region corresponding to nt 124–449 of the prototype HEV strain "Burma (M73218)".

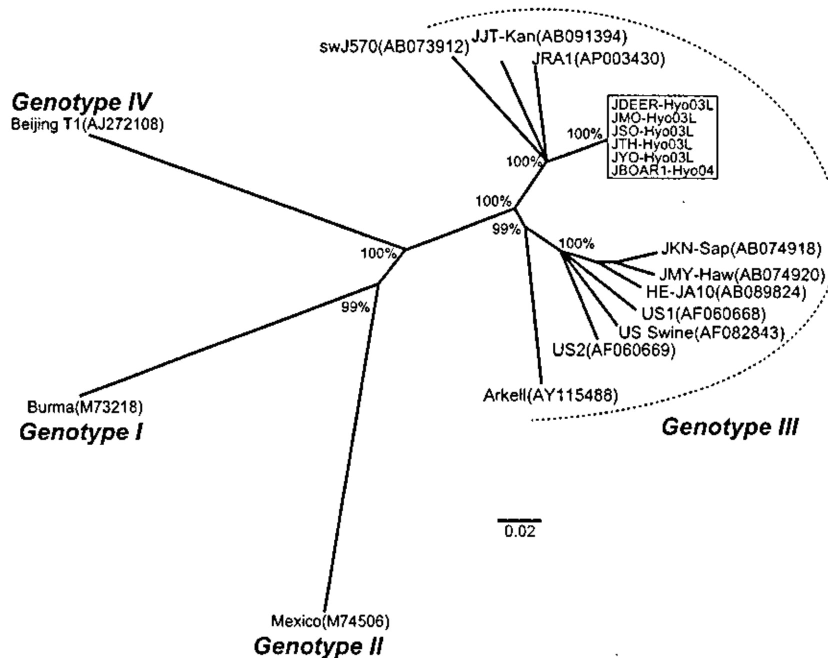

Fig. 3. Phylogenetic tree (NJ) with full or near-full-genome HEV isolates. Each of the genotypes I, II, and IV was represented by only one isolate, while the genotype III included all the isolates so far known for entire or near-entire genome sequence. The nodes with 1000-fold bootstrap values greater than 95% were indicated.

Experimental transmission studies have provided ample evidence of cross-species transmission of HEV and HEV-like virus: for example, infection of rat (Maneerat et al., 1996), swine (Meng et al., 1998), and macaque (van Cuyck-Gandre et al., 1998) with human HEV, and turkey with avian HEV (Sun et al., 2004). Despite the laboratory evidence, however, whether the interspecies transmission of HEV is really occurring between wild animals remains uncertain. Our observations in this study strongly suggests that it occurred between deer and boar in their wild life, because the HEV sequence from the boar (JBOAR1-Hyo04) and that from the deer (JDEER-Hyo03L) were almost identical.

Facing the extraordinary similarity of the sequences, one may argue that there might have been a cross-contamination between samples dealt with in this study. But, firstly, we have been very cautious to avoid cross-contamination between samples in our laboratory. Secondly, we found 19–23 mismatched nucleotides between the boar-derived HEV and the deer/patients-derived HEV, while only less than eight nucleotides were mismatching among the deer/patients-derived isolates, suggesting it is very unlikely that the boar-derived sequence would have been a contamination product of deer/patients-derived nucleic acids. Thirdly, we have reported that the mutation rate of HEV is estimated to be about 1.40×10^{-3} base substitutions per site per year (Takahashi et al., 2004). If calculated on this rate, the boar HEV must have diverged from the deer-HEV's progenitor strain about 2 years ago: The deer had been captured 1 year before the boar was done. Thus, the observed number of mismatched nucleotides is fairly better corresponding to an in vivo infection in the forest than to an in vitro contamination in our laboratory.

From deer to boar, or from boar to deer, which direction then? Our preliminary study indicated that HEV infection is endemic in the wild boar living in the forest of Hyogo prefecture, Japan: three out of seven boars hunted therefrom were positive not only for antibodies but also for RNA of HEV (Kitajima et al., in press), while only 1 of 100 deer hunted in the same forest tested positive for HEV RNA (our unpublished result). This suggests that boar must be the main reservoir of HEV while deer might merely be an occasional reservoir, infected from boar, in the habitat of these animals. Pigs are known to be one of the major reservoirs of HEV: almost 100% of Japanese pigs are reported to be positive for anti-HEV at 6 months of age (Takahashi et al., 2003b). Boar may be as susceptible as pig to HEV, since these animals are taxonomically fellow creatures.

In conclusion, it seems that boar and deer infect each other (more likely the former infects the latter) with HEV in their wild life in shared habitat, and serve as an HEV infection source for human beings, particularly for those who like *Gibier* cuisine. Japanese people, notorious as raw meat eaters, have an additional risk of acquiring HEV from wild animals.

Materials and methods

The materials and methods used in this study are briefly as follows.

The liver and serum specimens from boars were kept frozen until laboratory analyses. The boar, from which the JBOAR1-Hyo04 HEV isolate was obtained, was estimated to be less than 1 year of age, and weighed 6 kg. Nucleotide sequencing of the boar-derived HEV as well as those from the deer and the four patients was performed by the methods described previously (Takahashi et al., 2001, 2003a). Briefly, HEV RNA from the nucleic acids extracted from the liver or serum was reverse-transcribed to cDNA with use of the THERMOSCRIPT RT System (Invitrogen Corporation, CA, USA), and PCR amplification of seven overlapping regions of the HEV genome was carried out in the presence of PLATINUM Taq DNA Polymerase High Fidelity (Invitrogen). The 5′- and 3′-terminal sequences were amplified with 5′-Full RACE Core Set (TaKaRa Shuzo Co., Ltd., Shiga, Japan) and 3′ RACE System for Rapid Amplification of cDNA Ends (Invitrogen), respectively.

Acknowledgment

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References

Kitajima, N., Takahashi, K., Abe, N., Mishiro, S., 2004. HEV infection in wild boars in Japan. *Kanzo* 45, 557 (abstract in English).

Maneerat, Y., Clayson, E.T., Myint, K.S., Young, G.D., Innis, B.L., 1996. Experimental infection of the laboratory rat with the hepatitis E virus. J. Med. Virol. 48, 121–128.

Matsuda, H., Okada, K., Takahashi, K., Mishiro, S., 2003. Severe hepatitis E virus infection after ingestion of uncooked liver from a wild boar. J. Infect. Dis. 188, 944.

Meng, X.J., Halbur, P.G., Shapiro, M.S., Govindarajan, S., Bruna, J.D., Mushahwar, I.K., Purcell, R.H., Emerson, S.U., 1998. Genetic and experimental evidence for cross-species infection by swine hepatitis E virus. J. Virol. 72, 9714–9721.

Sun, Z.F., Larsen, C.T., Huang, F.F., Billam, P., Pierson, F.W., Toth, T.E., Meng, X.J., 2004. Generation and infectivity titration of an infectious stock of avian hepatitis E virus (HEV) in chickens and cross-species infection of turkeys with avian HEV. J. Clin. Microbiol. 42, 2658–2662.

Takahashi, K., Iwata, K., Watanabe, N., Hatahara, T., Ohta, Y., Baba, K., Mishiro, S., 2001. Full-genome nucleotide sequence of a hepatitis E virus strain that may be indigenous to Japan. Virology 287, 9–12.

Takahashi, K., Kang, J.-H., Ohnishi, S., Hino, K., Miyakawa, H., Miyakawa, Y., Maekubo, H., Mishiro, S., 2003a. Full-length sequences of six hepatitis E virus isolates of genotypes III and IV from patients with sporadic acute or fulminant hepatitis in Japan. Intervirology 46, 308–318.

Takahashi, M., Nishizawa, T., Miyajima, H., Gotanda, Y., Iita, T., Tsuda, F., Okamoto, H., 2003b. Swine hepatitis E virus strains in Japan form four phylogenetic clusters comparable with those of Japanese isolates of human hepatitis E virus. J. Gen. Virol. 84, 851–862.

Takahashi, K., Toyota, J., Karino, Y., Kang, J.-H., Maekubo, H., Abe, N., Mishiro, S., 2004. Estimation of the mutation rate of hepatitis E virus based on a set of closely related 7.5-year-apart isolates from Sapporo, Japan. Hepatol. Res. 29, 212–215.

Tamada, Y., Yano, K., Yatsuhashi, H., Inoue, O., Mawatari, F., Ishibashi, H., 2004. Consumption of wild boar linked to cases of hepatitis E. J. Hepatol. 40, 869–870.

Tei, S., Kitajima, N., Takahashi, K., Mishiro, S., 2003. Zoonotic transmission of hepatitis E virus from deer to human beings. Lancet 362, 371–373.

van Cuyck-Gandre, H., Cockman-Thomas, R., Caudill, J.D., Asher, L.S., Armstrong, K.L., Hauroeder, B., Clements, N.J., Binn, L.N., Longer, C.F., 1998. Experimental African HEV infection in cynomolgus macaques (*Macaca fascicularis*). J. Med. Virol. 55, 197–202.

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Prevalence of Hepatitis E Virus (HEV) Infection in Wild Boars and Deer and Genetic Identification of a Genotype 3 HEV from a Boar in Japan

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Zoonotic transmission of hepatitis E virus (HEV) from captured wild deer or boars to humans has been suggested. Antibody to HEV was detected in 9% of 35 wild boars and 2% of 117 wild deer tested, and a presumably indigenous HEV of genotype 3 was isolated from a boar in Japan.

Hepatitis E virus (HEV), the causative agent of hepatitis E, is an important human pathogen (4, 18, 21). The genome of HEV is approximately 7.2 kb in size and contains three open reading frames (ORF1, ORF2, and ORF3) (18). Although only one serotype has been recognized, extensive genomic diversity has been noted among HEV isolates, and HEV sequences have tentatively been classified into four genotypes (genotypes 1 to 4) (20). Transmission of HEV occurs primarily by the fecal-oral route through contaminated water supplies in developing countries (18). In addition, increasing evidence has indicated that hepatitis E is a zoonosis (4, 8, 10–13, 15, 16, 21, 24, 29). It has recently been suggested that zoonotic food-borne transmission of HEV from domestic pigs, wild boars, or wild deer to humans plays an important role in the occurrence of cryptic hepatitis E in Japan, where people have distinctive habits of ingesting raw fish (sushi or sashimi) and, less frequently, uncooked or undercooked meat (including the liver and colon or intestine of animals) (9, 23, 24, 29). The first animal strain of HEV to be isolated and characterized was a swine HEV from a pig in the United States in 1997 (10). Since then, many swine HEV isolates, which exhibit extensive genetic heterogeneity, have been identified worldwide and shown to be genetically closely related to strains of human HEV (1, 3, 5, 6, 16, 17, 25–27, 30). In previous studies, a high prevalence of the swine immunoglobulin G (IgG) class of antibody to HEV (anti-HEV) was found among 2- to 6-month-old Japanese pigs (58% or 1,448 of 2,500) (22), and a pair of Japanese swine and human HEV isolates of genotype 4 with 99% identity over the entire genome were identified (15). In addition, a certain proportion of packaged raw pig livers for sale in stores as food (1.9% or 7 of 363) were contaminated with HEV, which had high nucleotide sequence identity, up to 100%, with the HEV isolates recovered from Japanese patients with hepatitis E who had ingested undercooked pig liver before disease onset (29). As for HEV from wild boars, although Chandler et al. (1)

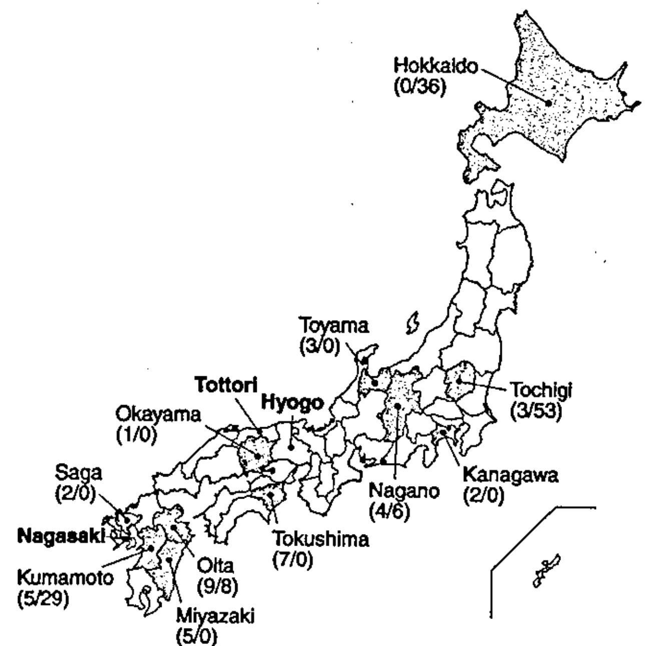

FIG. 1. Map of Japan showing the locations of Hokkaido Island and 10 prefectures where wild boars and/or deer were captured. The numbers before and after the slash in parentheses indicate the numbers of wild boars and of deer, respectively, examined in the indicated location. The three prefectures in boldface type are the prefectures in which patients with acute hepatitis E who contracted the disease after ingestion of uncooked or undercooked meat or liver from wild boars or deer have been reported (9, 23, 24).

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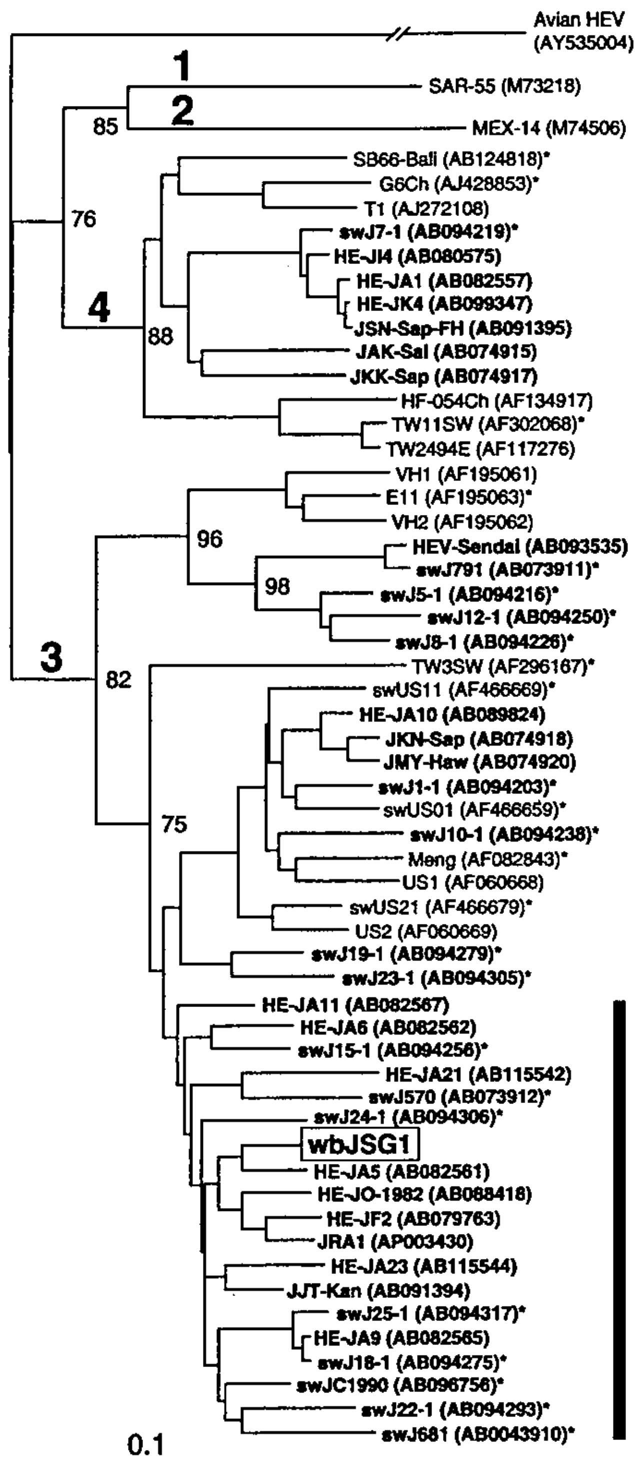

FIG. 2. Phylogenetic tree constructed by the neighbor-joining method (19) based on the partial nucleotide sequence (298 nt) of the ORF2 region of 56 human, swine, and boar HEV isolates, using an avian HEV (AY535004) as an outgroup. In addition to the wbJSG1 isolate obtained from a viremic boar in the present study, which is boxed for visual clarity, 55 representative human and swine HEV isolates whose common 298-nt sequences are known are included for comparison, and their accession numbers are shown in parentheses. The human, swine, and boar HEV strains isolated in Japan are indicated by boldface type. Asterisks denote swine HEV strains. A cluster consisting of the wbJSG1 strain isolated from a Japanese boar in the present study and 18 human and swine HEV strains isolated in Japan is indicated by a vertical bar. Bootstrap values of >70% are indicated for the major nodes as a percentage of the data obtained from 1,000 resamplings (2).

reported the prevalence of HEV antibody among captured wild pigs (boars) in Australia (25% or 15 of 59), boar HEV strains have not yet been identified. Furthermore, although HEV RNA was detected in a leftover portion of deer meat that was implicated in the development of food-borne hepatitis E (24), the prevalence of HEV infection among wild deer remains unknown.

Therefore, in the present study, we obtained and analyzed paired serum and liver specimens, serum only, or liver tissues only from 41 wild boars (*Sus scrofa leucomystax*) that had been captured in Tochigi, Toyama, Nagano, Kanagawa, and Okayama Prefectures on mainland Honshu, Tokushima Prefecture on Shikoku Island, and Saga, Oita, Kumamoto, and Miyazaki Prefectures on Kyushu Island (listed by location from north to south in Japan) between December 2002 and February 2003 and between December 2003 and March 2004. We tested 132 wild deer (Sika deer; *Cervus nippon*) that had been caught on Hokkaido Island (*C. nippon yesoensis*), in Tochigi and Nagano Prefectures on mainland Honshu (*C. nippon centralis*), and in Oita and Kumamoto Prefectures on Kyushu Island (*C. nippon nippon*) between October 2003 and March 2004 (Fig. 1). A total of 35 serum samples and 33 liver tissues, including 27 paired serum and liver specimens, were available from the 41 boars, and 117 serum samples and 132 liver tissues, including 117 paired serum and liver specimens, were available from the 132 deer. The serum samples were tested for the presence of anti-HEV IgG by in-house enzyme-linked immunosorbent assay using purified recombinant ORF2 protein that had been expressed in the pupae of silkworms (14) as the antigen probe as described previously (22), with slight modifications. For the boar anti-HEV IgG assay, the peroxidase-conjugated rabbit IgG fraction to swine IgG (whole molecule) (ICN/Cappel, Aurora, Ohio) was used as described previously for the swine anti-HEV IgG assay (22), and for the deer anti-HEV IgG assay, peroxidase-labeled affinity-purified antibody to deer IgG (H+L; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) was used instead of the enzyme-labeled anti-swine IgG antibodies. The serum samples and liver tissues were tested for the presence of HEV RNA by reverse transcription (RT)-PCR by the method described previously, with primers targeting the ORF2 region (14). To confirm the presence of HEV RNA, a part of the ORF1 region was amplified by nested RT-PCR (14). The two RT-PCR assays used had the capability to amplify all four known genotypes of HEV (14, 22, 29). The amplified products were sequenced directly on both strands.

The A_{450} value of boar anti-HEV antibodies ranged from 0.003 to 0.866, and 3 (9%) of the 35 serum samples had an A_{450} value of $\geq$0.300. The A_{450} values of these three samples (0.335, 0.655, and 0.866) decreased to less than 30% of the original values after absorption with the same recombinant ORF2 protein that was used as the antigen probe, but they remained greater than 70% of the original values after absorption with a mock protein obtained from the pupae of silkworms infected

with nonrecombinant baculovirus, confirming the specificity of the assay. Therefore, these three serum samples from boars in Nagano Prefecture (two samples) and Miyazaki Prefecture (one sample) were conservatively regarded as being positive for boar anti-HEV IgG in the present study. The A_{450} value of deer anti-HEV IgG ranged from 0.012 to 1.442, and 2 (2%) of the 117 serum samples had A_{450} values of $\geq$0.300 (0.591 and 1.442); the specificity was confirmed by the absorption assay. Deer anti-HEV IgG was detectable in 1 (3%) of the 32 serum samples from deer in Hokkaido and 1 (2%) of the 53 samples from deer in Tochigi Prefecture.

Among all of the serum and liver specimens from the boars and deer, HEV RNA was reproducibly detected in paired serum and liver specimens obtained from a male boar with a body weight of 60 kg that had been caught in Saga Prefecture on Kyushu Island on 19 December 2003, although the viremic boar was negative for anti-HEV IgG and had no clinical manifestations. The HEV sequences amplified from the serum and liver tissue of the infected boar were 100% identical in both a 412-nucleotide (nt) sequence of the ORF2 region and another 412-nt sequence of the ORF1 region (accession no. AB180052 to AB180055). The HEV isolate (wbJSG1) obtained in the present study was close to known human and swine genotype 3 isolates, with 82.9 to 93.9% identity in the 412-nt ORF2 sequence, and was most closely related to the HE-JA5 isolate of genotype 3 which is presumed to be indigenous to Japan (14). The phylogenetic tree constructed by the neighbor-joining method (19) based on the partial ORF2 sequence of 298 nt confirmed that the wbJSG1 isolate belonged to genotype 3, and it segregated into a cluster consisting of 18 HEV strains that had been isolated from 10 Japanese patients with no history of travel to countries where the virus is endemic who developed sporadic acute or fulminant hepatitis (14, 29) and from eight Japanese farm pigs (16, 22) (Fig. 2). Among HEV strains recovered from patients who developed hepatitis E after ingestion of uncooked or undercooked meat or liver from wild boars or deer for which the ORF2 sequences were not available, the wbJSG1 isolate obtained in the present study was 90.2 to 90.9% identical in the 317-nt ORF1 sequence to the HEV isolates of genotype 3 (AY427956 and AY427957) recovered from two patients who had ingested grilled meat from wild boars in Nagasaki Prefecture (23) and only 87.4 to 87.7% identical in the 326-nt ORF1 sequence to the HEV isolates of genotype 3 (AB114179 to AB114183) recovered from four patients with hepatitis E who had consumed raw meat from a wild deer in Hyogo Prefecture and from a leftover portion of the deer meat that had been kept frozen to be eaten in the future (24). In addition, the wbJSG1 isolate was merely 77.5% similar in the 326-nt ORF1 sequence to the JSF-Tot03 isolate of genotype 4 (AB114178) that had been recovered from a patient who had eaten uncooked liver from a wild boar in Tottori Prefecture (9), suggesting that heterogeneous strains of HEV of genotype 3 or 4 are circulating among wild boars and deer in Japan. Based on the finding that one of the two wild boars caught in Saga Prefecture in the present study was HEV viremic, meat and liver from the wbJSG1-infected boar were not ingested, thereby possibly preventing food-borne transmission of HEV.

Prevalence of anti-HEV IgG in pigs is usually age dependent; swine HEV RNA can often be detected in pigs 2 to 4 months of age but is less commonly detected in older pigs (16, 22, 28). The HEV-infected boar identified in the present study weighed 60 kg, suggesting that the viremic boar was approximately 2 years of age and that wild boars can acquire de novo HEV infection at older ages than domestic pigs. However, it is difficult to estimate ages of wild boars and deer living under natural conditions based on their body weights. It has been reported that periodic growth incremental lines found universally in dental hard tissues allow for reliable estimation of age in wild animals (7). Therefore, extended studies must be undertaken to investigate the seroprevalence of HEV and frequency of viremia in wild deer and boars in relation to their ages estimated by use of tooth increments.

The results obtained in the present study indicate that wild boars and deer in Japan are infected with HEV, although at much lower rates than domestic pigs in Japan, and that a certain proportion of wild boars in Japan are HEV viremic and may act as sources of HEV infection in humans. The isolation of a domestic HEV strain from a Japanese wild boar with high nucleotide sequence identity to human HEV in Japan provides further evidence for zoonotic food-borne transmission of HEV from wild boars to humans. Further extended studies are required to fully elucidate the epidemiology of HEV infection in animals and possible zoonotic transmission in an attempt to prevent cryptic hepatitis E occurring not only in industrialized countries but also in developing countries.

Nucleotide sequence accession numbers. The sequences determined in the present study have been deposited in the DDBJ, GenBank, and EMBL nucleotide databases under the following accession numbers: AB180052 for the ORF1 sequence of the wbJSG1 isolate recovered from the liver of the HEV-infected boar (ORF1, liver), AB180053 (ORF2, liver), AB180054 (ORF1, serum), and AB180055 (ORF2, serum).

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REFERENCES

1. **Chandler, J. D., M. A. Riddell, F. Li, R. J. Love, and D. A. Anderson.** 1999. Serological evidence for swine hepatitis E virus infection in Australian pig herds. Vet. Microbiol. 68:95–105.
2. **Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791.
3. **Garkavenko, O., A. Obriadina, J. Meng, D. A. Anderson, H. J. Bernard, B. A. Schroeder, Y. E. Khudyakov, H. A. Fields, and M. C. Croxson.** 2001. Detection and characterization of swine hepatitis E virus in New Zealand. J. Med. Virol. 65:525–529.
4. **Harrison, T. J.** 1999. Hepatitis E virus—an update. Liver 19:171–176.
5. **Hsieh, S.-Y., X.-J. Meng, Y.-H. Wu, S.-T. Liu, A. W. Tam, D.-Y. Lin, and Y.-F. Liaw.** 1999. Identity of a novel swine hepatitis E virus in Taiwan forming a monophyletic group with Taiwan isolates of human hepatitis E virus. J. Clin. Microbiol. 37:3828–3834.

6. **Huang, F. F., G. Haqshenas, D. K. Guenette, P. G. Halbur, S. K. Schommer, F. W. Pierson, T. E. Toth, and X. J. Meng.** 2002. Detection by reverse transcription-PCR and genetic characterization of field isolates of swine hepatitis E virus from pigs in different geographic regions of the United States. J. Clin. Microbiol. **40:**1326–1332.
7. **Iinuma, Y. M., S. Tanaka, K. Kawasaki, T. Kuwajima, H. Nomura, M. Suzuki, and N. Ohtaishi.** 2004. Dental incremental lines in Sika deer (*Cervus nippon*); polarized light and fluorescence microscopy of ground sections. J. Vet. Med. Sci. **66:**665–669.
8. **Kabrane-Lazizi, Y., J. B. Fine, J. Elm, G. E. Glass, H. Higa, A. Diwan, C. J. Gibbs, Jr., X.-J. Meng, S. U. Emerson, and R. H. Purcell.** 1999. Evidence for widespread infection of wild rats with hepatitis E virus in the United States. Am. J. Trop. Med. Hyg. **61:**331–335.
9. **Matsuda, H., K. Okada, K. Takahashi, and S. Mishiro.** 2003. Severe hepatitis E virus infection after ingestion of uncooked liver from a wild boar. J. Infect. Dis. **188:**944.
10. **Meng, X.-J., R. H. Purcell, P. G. Haubur, J. R. Lehman, D. M. Webb, T. S. Tsareva, J. S. Haynes, B. J. Thacker, and S. U. Emerson.** 1997. A novel virus in swine is closely related to the human hepatitis E virus. Proc. Natl. Acad. Sci. USA **94:**9860–9865.
11. **Meng, X.-J., P. G. Halbur, M. S. Shapiro, S. Govindarajan, J. D. Bruna, I. K. Mushahwar, R. H. Purcell, and S. U. Emerson.** 1998. Genetic and experimental evidence for cross-species infection by swine hepatitis E virus. J. Virol. **72:**9714–9721.
12. **Meng, X. J., B. Wiseman, F. Elvinger, D. K. Guenette, T. E. Toth, R. E. Engle, S. U. Emerson, and R. H. Purcell.** 2002. Prevalence of antibodies to hepatitis E virus in veterinarians working with swine and in normal blood donors in the United States and other countries. J. Clin. Microbiol. **40:**117–122.
13. **Meng, X. J.** 2003. Swine hepatitis E virus: cross-species infection and risk in xenotransplantation. Curr. Top. Microbiol. Immunol. **278:**185–216.
14. **Mizuo, H., K. Suzuki, Y. Takikawa, Y. Sugai, H. Tokita, Y. Akahane, K. Itoh, Y. Gotanda, M. Takahashi, T. Nishizawa, and H. Okamoto.** 2002. Polyphyletic strains of hepatitis E virus are responsible for sporadic cases of acute hepatitis in Japan. J. Clin. Microbiol. **40:**3209–3218.
15. **Nishizawa, T., M. Takahashi, H. Mizuo, H. Miyajima, Y. Gotanda, and H. Okamoto.** 2003. Characterization of Japanese swine and human hepatitis E virus isolates of genotype IV with 99% identity over the entire genome. J. Gen. Virol. **84:**1245–1251.
16. **Okamoto, H., M. Takahashi, T. Nishizawa, K. Fukai, U. Muramatsu, and A. Yoshikawa.** 2001. Analysis of the complete genome of indigenous swine hepatitis E virus isolated in Japan. Biochem. Biophys. Res. Commun. **289:** 929–936.
17. **Pina, S., M. Buti, M. Cotrina, J. Piella, and J. Girones.** 2000. HEV identified in serum from humans with acute hepatitis and in sewage of animal origin in Spain. J. Hepatol. **33:**826–833.
18. **Purcell, R. H., and S. U. Emerson.** 2001. Hepatitis E virus, p. 3051–3061. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, M. A. Martin, R. A. Lamb, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, Pa.
19. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
20. **Schlauder, G. G., and I. K. Mushahwar.** 2001. Genetic heterogeneity of hepatitis E virus. J. Med. Virol. **65:**282–292.
21. **Smith, J. L.** 2001. A review of hepatitis E virus. J. Food Prot. **64:**572–586.
22. **Takahashi, M., T. Nishizawa, H. Miyajima, Y. Gotanda, T. Iita, F. Tsuda, and H. Okamoto.** 2003. Swine hepatitis E virus strains in Japan form four phylogenetic clusters comparable with those of Japanese isolates of human hepatitis E virus. J. Gen. Virol. **84:**851–862.
23. **Tamada, Y., K. Yano, H. Yatsuhashi, O. Inoue, F. Mawatari, and H. Ishibashi.** 2004. Consumption of wild boar linked to cases of hepatitis E. J. Hepatol. **40:**869–870.
24. **Tei, S., N. Kitajima, K. Takahashi, and S. Mishiro.** 2003. Zoonotic transmission of hepatitis E virus from deer to human beings. Lancet **362:**371–373.
25. **Wang, Y., H. Zhang, N. Xia, G. Peng, H. Lan, H. Zhuang, Y. Zhu, S. Li, K. Tian, W. Gu, J. Lin, X. Wu, H. Li, and T. J. Harrison.** 2002. Prevalence, isolation, and partial sequence analysis of hepatitis E virus from domestic animals in China. J. Med. Virol. **67:**516–521.
26. **Wibawa, I. D. N., D. H. Muljono, Mulyanto, I. G. A. Suryadarma, F. Tsuda, M. Takahashi, T. Nishizawa, and H. Okamoto.** 2004. Prevalence of antibodies to hepatitis E virus among apparently healthy humans and pigs in Bali, Indonesia: identification of a pig infected with a genotype 4 hepatitis E virus. J. Med. Virol. **73:**38–44.
27. **Wu, J. C., C. M. Chen, T. Y. Chiang, I. J. Sheen, J. Y. Chen, W. H. Tasi, Y. H. Huang, and S. D. Lee.** 2000. Clinical and epidemiological implications of swine hepatitis E virus infection. J. Med. Virol. **60:**166–171.
28. **Wu, J. C., C. M. Chen, T. Y. Chiang, W. H. Tsai, W. J. Jeng, I. J. Sheen, C. C. Lin, and X. J. Meng.** 2002. Spread of hepatitis E virus among different-aged pigs: two-year survey in Taiwan. J. Med. Virol. **66:**488–492.
29. **Yazaki, Y., H. Mizuo, M. Takahashi, T. Nishizawa, N. Sasaki, Y. Gotanda, and H. Okamoto.** 2003. Sporadic acute or fulminant hepatitis E in Hokkaido, Japan, may be food-borne, as suggested by the presence of hepatitis E virus in pig liver as food. J. Gen. Virol. **84:**2351–2357.
30. **Yoo, D., P. Willson, Y. Pei, M. A. Hayes, A. Deckert, C. E. Dewey, R. M. Friendship, Y. Yoon, M. Gottschalk, C. Yason, and A. Giulivi.** 2001. Prevalence of hepatitis E virus antibodies in Canadian swine herds and identification of a novel variant of swine hepatitis E virus. Clin. Diagn. Lab. Immunol. **8:**1213–1219.

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XENOTRANSPLANTATION

Molecular investigation of hepatitis E virus infection in domestic and miniature pigs used for medical experiments*

Tanaka H, Yoshino H, Kobayashi E, Takahashi M, Okamoto H. Molecular investigation of hepatitis E virus infection in domestic and miniature pigs used for medical experiments.
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*The nucleotide sequence data reported in this study have been assigned DDBJ/GenBank/EMBL accession numbers AB177355 to AB177376.

Abstract: Background: Hepatitis E virus (HEV) infection is highly prevalent among domestic pigs in Japan. It has been reported that pig handlers such as farmers and veterinarians are at increased risk of contracting HEV infection. Pigs are regarded as the most acceptable candidate animals for xenotransplantation and, recently, they are being used as experimental animals.
Methods: We investigated the prevalence of IgG class antibodies to swine HEV (anti-HEV) and HEV RNA among 152 2-month-old domestic pigs and 38 miniature pigs of 4 to 10 months of age that had been brought to our center for medical experiments from five swine farms (A–E) in Japan. Serum samples were tested for anti-HEV by in-house enzyme immunoassay, and for HEV RNA by reverse transcription-polymerase chain reaction using primers targeting the open reading frame 2 (ORF2) region.
Results: One percent (one of 84), 6% (one of 16), and 38% (20 of 52) of the domestic pigs from farms A, B and C, respectively, had detectable HEV RNA, and the 22 HEV isolates recovered from the viremic pigs were 89.8 to 100% identical to each other in the 412-nucleotide sequence of ORF2 and segregated into three clusters within genotype 3. Although one pig from farm A had detectable HEV RNA reproducibly, the HEV isolate recovered from this pig was up to 100% similar to those recovered from pigs from farm C, and the sera from all 84 pigs from farm A were negative for anti-HEV. These results suggested that farm A is free from HEV infection. As the viremic pig from farm A had been raised for 1 month in a barn at our center before serum sampling, it is most likely that the pig acquired HEV infection in the barn at our center where HEV-viremic pigs from farm C had been reared for several days approximately 3 months earlier. The 38 miniature pigs from farms D and E were negative for both anti-HEV and HEV RNA. In an attempt to further investigate the prevalence of HEV infection, pigs that were being raised in four swine farms (farms A, C, D, and E) were tested for anti-HEV. Although 96 (86%) of the 112 pigs from farm C were positive for anti-HEV, none of the 48 pigs in farm A and 138 miniature pigs in farms D and E was positive for anti-HEV.
Conclusions: These results suggest that three of the five swine farms tested were free from HEV, and that periodic testing for anti-HEV and HEV RNA of pigs used as experimental animals and pigs raised in swine farms from which pigs are purchased, is useful for providing HEV-free pigs to researchers who are engaged in studies using pigs.

Key words: experimental animals – hepatitis E virus – HEV RNA – phylogenetic tree – pigs – zoonosis

Abbreviations: anti-HEV: antibodies to HEV; ELISA: enzyme-linked immunosorbent assay; HEV: hepatitis E virus; PCR: polymerase chain reaction; RT-PCR: reverse transcription-polymerase chain reaction; SPF: specific pathogen-free.

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Introduction

Hepatitis E virus (HEV), which has a single-stranded, positive-stranded RNA genome of approximately 7200 nucleotides, is one of the major causative agents of acute hepatitis in many developing countries in Asia, Africa and Central America. HEV is transmitted via the fecal-oral route [1]. Recently, evidence is accumulating that HEV-associated hepatitis is a zoonosis and cross-species infection of HEV has been documented [2–9]. HEV is much more diverse and widespread than previously thought. To date, four major genotypes (1 to 4) of HEV have been identified [1,10,11].

In Japan, stray dogs captured by public health centers have been used for medical experiments in experimental animal centers at research institutions. Recently, however, there have been concerns about the welfare and care of such dogs used in medical experiments. It has been noted that pigs, which have an anatomy and physiology similar to those of humans, may be used in animal studies instead of dogs or as a resource of organs for xenotransplantation [5,12,13].

The antibody to HEV (anti-HEV) has been detected among pigs in developing countries including Nepal [14] and Thailand [15] and among pigs in industrialized countries including the United States [7], Canada [15], Korea [15], Taiwan [3,16], and Australia [17]. Swine HEV, which was recently isolated from pigs, is antigenically and genetically closely related to the human HEV [4,18–20]. HEV infection in pigs generally occurs at 2 to 3 months of age [21–24], and approximately 80 to 100% of pigs in Japan and the United States are transiently infected [7,23].

It was found that all 11 pig handlers who were tested in China were positive for anti-HEV immunoglobulin G (IgG), although 17 (55%) of 31 apparently healthy blood donors in the same geographic region were positive for anti-HEV IgG [15]. In Thailand, five of the seven pig handlers who were tested were seropositive for anti-HEV IgG [15]. To assess the potential risk of zoonotic HEV infection, several sero-epidemiological studies on pig handlers have recently been conducted in industrialized countries. In Taiwan, Hsieh et al. [3] found that 27% of the Taiwanese pig handlers who were tested were positive for anti-HEV IgG, whereas only about 8% of the control subjects were seropositive. In the United States, the prevalence of HEV infection among North Carolina swine workers (11%) was 4.5-fold higher than that among non-swine workers (2.4%) [25]. Upon testing veterinarians working with swine and normal blood donors in the United States for anti-HEV IgG, there was a difference in the prevalence of anti-HEV in both swine veterinarians and blood donors among the eight selected states in the United States [26]. The prevalence of anti-HEV among the swine veterinarians was higher than that among the blood donors [26]. These findings suggest that, like pig handlers, researchers using pigs as experimental animals are at risk for zoonotic HEV infection. In Japan, patients with domestically infected fulminant hepatitis E who subsequently died of hepatic failure have been reported [9,27], suggesting that HEV infection is a serious zoonotic disease.

The aims of the present study were to find pig farms that are free of HEV and to offer this information to researchers who conduct experiments using pigs. We tested pigs that had been brought to the animal center of our institute for the presence of HEV RNA and anti-HEV, and investigated the prevalence of HEV infection among pigs that were being raised on five pig farms with which we had made contracts to purchase pigs.

Materials and methods

Sera from the experimental animals

This study included serum samples that had been obtained from a total of 312 domestic pigs and 176 miniature pigs during the period from June 2002 to February 2004. Domestic pigs of 2 months of age (body weight: 20 to 30 kg) were brought to our center from three pig farms (A, B and C) with which we had made contracts to purchase domestic pigs. Farms A and B are under one-line management of production and breeding by 30 or 40 sows and produce approximately 700 fattened pigs per year. Farm C is a pig-fattening farm and fattens approximately 7500 pigs provided by four pig-producing farms (C1, C2, C3 and C4) per year. Two-month-old domestic pigs that had been produced in farms C1 and C2 were brought to our center after being fattened in farm C. Of note, the pigs from farms C1, C2, C3 and C4 were raised separately in farm C. Farms A, B, C, C1, C2, C3, and C4 are all privately managed farms, where pigs are raised in conventional housing facilities and are free of foot-and-mouth disease, hog cholera, swine vesicular disease and African swine fever.

Serum samples were obtained from 84, 16, and 52 pigs that had been brought to our center from farms A, B and C, respectively. All of these serum samples were tested for the presence of swine anti-HEV IgG and HEV RNA. In addition, serum samples were collected from 48 3- to 6-month-old

domestic pigs in farm A and 112 pigs of 4 or 5 months of age that were fattened in farm C (27 sera had been derived from farm C1, 26 from farm C2, 29 from farm C3 and 30 from farm C4), and were tested for swine anti-HEV IgG for the purpose of conducting a serosurvey of HEV infection in these swine farms.

Thirty-eight miniature pigs of 4 to 10 months of age (body weight: 15 to 25 kg) were brought to our center from two farms (D and E), and serum samples from all of these miniature pigs were tested for the presence of swine anti-HEV IgG and HEV RNA. Farm D is an enterprise with the one-line management of production and breeding by 54 sows of miniature pigs (Clawn), and it produces approximately 500 miniature pigs per year for use as experimental animals. Farm E is managed by the Japanese government and is under the one-line management by the breeding of 91 sows of miniature pigs (Mexican Hairless Pig), and it produces 267 miniature pigs per year for conservation of miniature pigs. The pigs in farm E are bred under the specific pathogen-free (SPF) condition, and the pigs in farm D are raised under conditions similar to those for SPF pigs in confinement housing facilities. Serum samples from 138 additional miniature pigs aged 4 to 7 months that were reared in farms D and E were tested for swine anti-HEV IgG for the purpose of conducting a serosurvey of HEV infection in the farms.

A total of 190 pigs that had been brought to our center from farms A, B, C, D and E were used for medical experiments including pharmacokinetic studies, endoscopic and angiographic examinations, and surgical operations such as abdominal or open heart surgery and transplantation of porcine liver, kidney, or intestine.

Enzyme-linked immunosorbent assay for detection of anti-HEV antibodies

To detect swine anti-HEV IgG, enzyme-linked immunosorbent assay (ELISA) was performed using purified recombinant open reading frame 2 (ORF2) protein of the HE-J1 strain of HEV (genotype 4) that had been expressed in the pupae of silkworm, according to the method described previously [23]. The absorbance of each sample was read at 450 nm. The cut-off value used for the swine anti-HEV IgG assay was 0.366, as described previously [23, 28]. The specificity of the anti-HEV assay was verified by absorption with the same recombinant ORF2 protein that was used as the antigen probe or a mock protein obtained from the pupae of silkworm infected with non-recombinant baculovirus. Briefly, when the absorbance value of the tested sample was < 30% of the original value after absorption with the recombinant ORF2 protein and was > 70% of the original value after absorption with a mock protein, the sample was considered to be positive for anti-HEV.

Detection of HEV RNA

Reverse transcription-polymerase chain reaction (RT-PCR) was performed for detection of HEV RNA. Total RNA was extracted from 100 μl of swine serum, reverse-transcribed, and subjected to nested PCR with ORF2 primers, as described previously [28]. The size of the amplification product of the first-round PCR was 506 bp and that of the second-round PCR was 457 bp. The nested RT-PCR assay was performed in duplicate and reproducibility was confirmed.

Sequence analysis of PCR products

The amplification products were sequenced directly on both strands using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using Genetyx-Mac version 12.2.0 (Software Development, Tokyo, Japan) and ODEN version 1.1.1 from the DNA Data Bank of Japan (DDBJ; National Institute of Genetics, Mishima, Japan) [29]. Sequence alignments were generated by CLUSTAL W (version 1.8) [30]. Phylogenetic trees were constructed by the neighbor-joining method [31] based on the partial nucleotide sequence of the ORF2 region (412 nucleotides). Bootstrap values were determined on 1000 resamplings of the data sets [32]. The final tree was obtained using the Tree View program (version 1.6.6) [33].

Results

Prevalence of swine anti-HEV IgG and HEV RNA among pigs used for medical experiments

This study included a total of 152 2-month-old domestic pigs (20 to 30 kg) and 38 4- to 10-month-old miniature pigs (15 to 25 kg) that had been brought to our center from five pig farms. Serum samples were obtained at the time that the pigs were used for medical experiments. One 2-month-old domestic pig was used for experiments 1 month after being transferred to our center (i.e. when it was 3 months old), while the remaining domestic pigs were used for experiments within 1 week after being transferred to our center. Although all of the