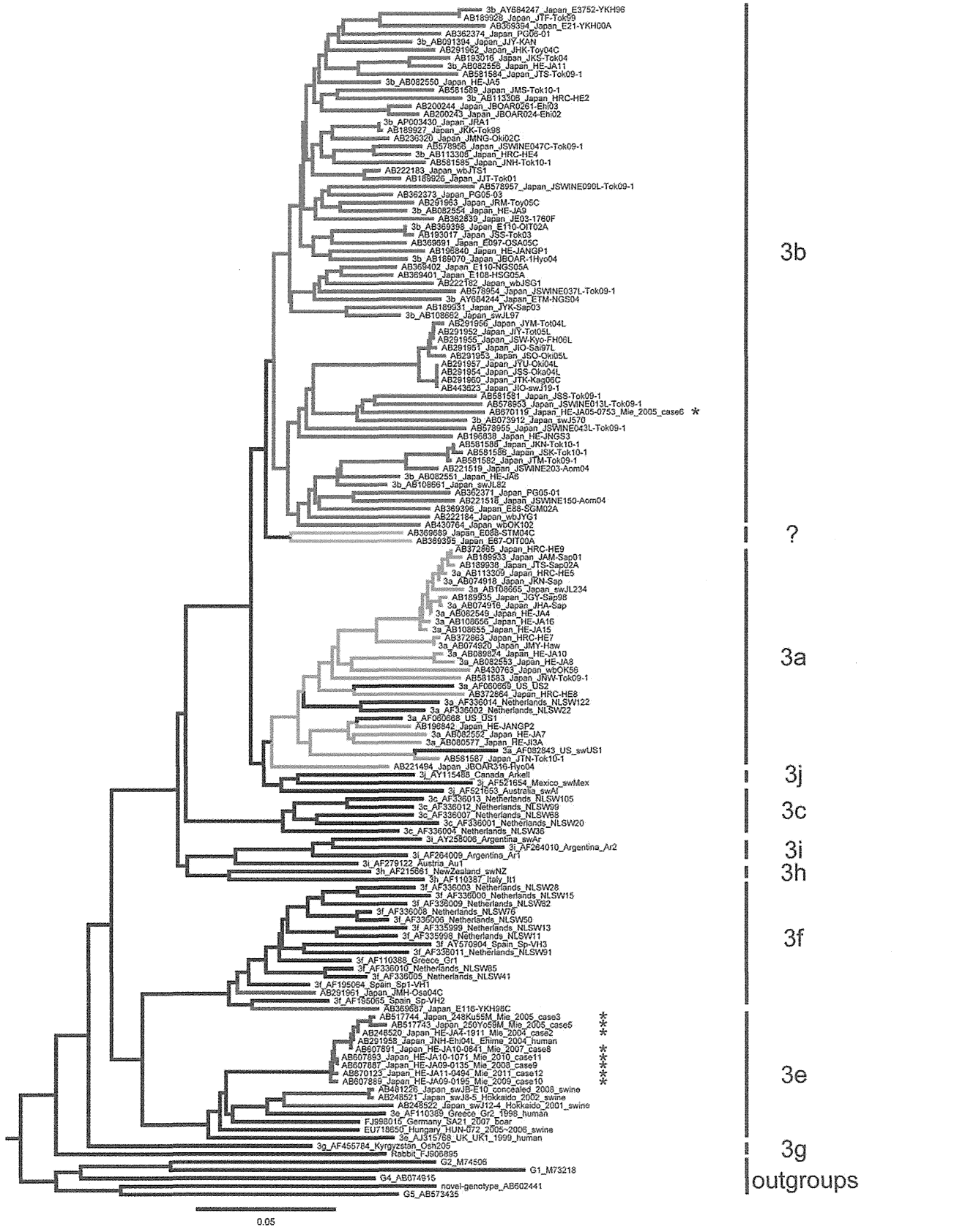

Fig. 3. The neighbor-joining tree of 287-nt sequence alignment within ORF1 containing 104 genotype 3 strains isolated in Japan and outgroup strains. Sequences in the literature of Lu et al. are included as guides for subtyping (Lu et al., 2006). Subtypes of those sequences are shown at the top of the strain names. The ancestral lineage states of clades are indicated using a parsimony approach and the colors are consistent with Fig. 2. The origin of each strain is also indicated by the strain names. The strains isolated in Mie prefecture are indicated by $*$ following the strain name. The scale bar indicates nucleotide substitutions·site^{-1}.

Table 2
TMRCAs of all of Japan-indigenous 3e and Mie and Ehime 3e strains (Median and 95% credible interval).

Clock		Strict clock	Relaxed uncorrelated clock	
			Log normal	Exponential
Clock rate*		$1.34\ (1.02–1.69) \times 10^{-3}$	$1.34\ (1.02–1.69) \times 10^{-3}$	$1.49\ (1.03–2.02) \times 10^{-3}$
TMRCAs	All	1963 (1944–1974)	1964 (1948–1974)	1966 (1946–1982)
	Mie and Ehime	2003 (2001–2004)	2003 (2001–2004)	2002 (1999–2004)

* Substitution/site/year.

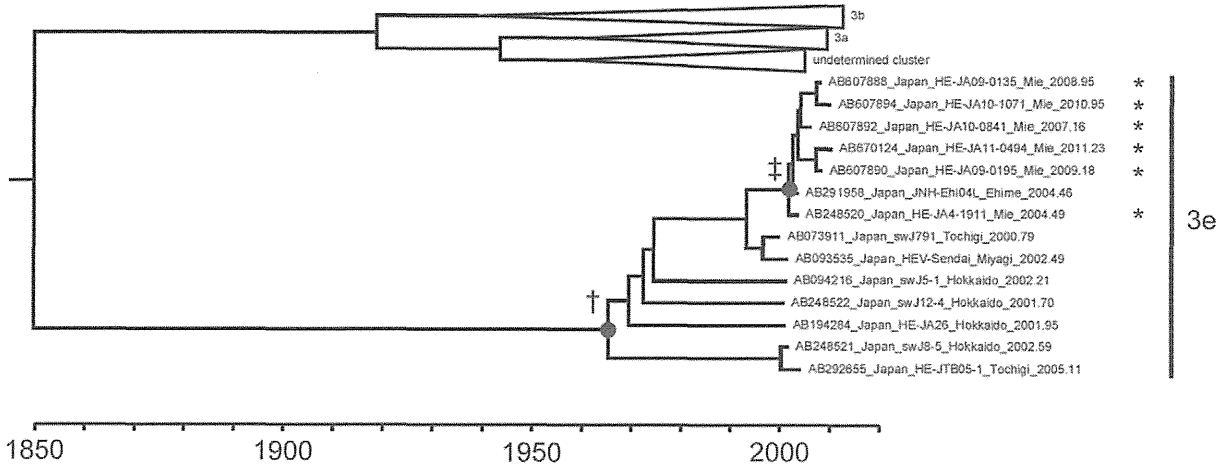

Fig. 4. The maximum clade support phylogeny for the dated Japanese sequence set of ORF2 region (412 nt). Japan-indigenous 3b, 3a, and undetermined clusters are collapsed, and the detail is shown in Supplemental Fig. 1. The node with "†" unifies all of the Japan-indigenous 3e strains. The node with "‡" integrates Mie and Ehime 3e strains. The strains isolated in Mie prefecture are indicated by * following the strain names. The branch lengths and node heights are in units of years.

4. Discussion

Genotype 3 HEV strains has been reported from throughout the world, including countries in Europe, Asia, New World, Oceania, and Africa (Kaba et al., 2010; Lu et al., 2006), and have been tried to be divided into subtypes, namely, 3a–j (Lu et al., 2006). In Japan, genotype 3 is the most prevalent genotype infecting humans (Sakata et al., 2008; Takahashi et al., 2010b) as well as swine (Takahashi et al., 2003). The existence of at least three lineages of Japanese genotype 3 strains, namely, 3us, 3jp, and 3sp, has been reported (Takahashi et al., 2003). By comparing the review by Lu et al. and the report by Takahashi et al., the data suggest that the Japanese lineages 3us, 3jp, and 3sp correspond to 3a, 3b, and 3e, respectively. In the present study, we used the Hepatitis Virus Database to collect many HEV genotype 3 strains isolated in Japan. By studying the phylogenetic trees of two genomic regions, 301 nt-ORF2 and 287 nt-ORF1, we found that Japan-indigenous genotype 3 strains were divided into two major subtypes or lineages 3a and 3b, the comparatively minor lineage 3e, and several unassigned strains. However, the separation between 3a, 3b, and the unassigned strains was obscure, and for definite subtyping, more sensitive phylogenetic analyses using longer sequences are needed. The 3e cluster, however, was clearly segregated, with sufficient bootstrap values even in the short region trees of ORF2 and ORF1 when only Japanese strains were included (data not shown).

Japan-indigenous 3e strains that have been reported from other areas in Japan except Mie prefecture are indicated in Figs. 1–3. The phylogenetic tree of the short ORF2 region (301 nt) suggests that at least 3 lineages of 3e strains had intruded into Japan (Fig. 2a). Several strains were isolated from swine, but the areas where the pig farms were located were concealed for ethical reasons to protect pig breeders from damaging publicity (Sapsutthipas et al., 2009; Urayama et al., 2010). Phylogenetic analysis separated one of the swine strains, namely, swJB-L8 (Sapsutthipas et al., 2009), from the other Japan-indigenous 3e lineages (Fig. 2a). Another lineage contains several strains isolated from pig farms (swJ5, swJ8, swJ12) in Hokkaido (Takahashi et al., 2003), one strain isolated from an acute hepatitis patient (HE-JA26) in Hokkaido (Mizuo et al., 2005), and one strain isolated from a blood donor (HE-JTB05-1) in Tochigi prefecture (Fukuda et al., 2007), which is one of the prefectures in Honshu, distant from Mie prefecture (Figs. 1a and 2a). Several swine strains isolated in unknown locations (swJB-E6, swJB-E10, swJB-F1, and swJB-D1) were also included in this lineage (Sapsutthipas et al., 2009). Another Japan-indigenous 3e lineage contained all the Mie 3e strains (Fig. 2a), one strain isolated from swine (swJ791) in Tochigi prefecture (Okamoto et al., 2001), and one strain isolated from an acute hepatitis patient (HEV-Sendai) in Miyagi prefecture (Inoue et al., 2009a), which is another Honshu prefecture that is distant from Mie prefecture (Figs. 1a–2a). The two strains, swJ791 and HEV-Sendai, belonged to the same lineage as the Mie 3e strains, but the homology with Mie 3e strains was only 97.3–98.1%. On the other hand, the strain most closely related to the Mie strains was JNH-Ehi04L, which was isolated from a patient with acute hepatitis in Ehime prefecture in Shikoku (Inoue et al., 2006a), another distant location from Mie prefecture (Fig. 1a). The homology between this strain and the Mie strains ranged from 99.0% to 99.5%. It is difficult to explain why this strain was isolated in Ehime prefecture, since the patient did not travel to or consume food from Mie prefecture. Of note, the strain was isolated in a laboratory different from that associated with the Mie strains, so cross contamination between the strains is impossible.

In the 3e cluster (Fig. 2a), 3 lineages of Japanese strains were intricately intertwined with some clusters from European countries. However, individual Japanese strains did not show a direct close relationship with individual European strains. This observation suggests that Japanese 3e lineages (not strains) are closely related to the European lineages (not strains). The presence of old

phylogenetic nodes that connect the Japanese and European lineages (Fig. 2a and Fig. 3) suggests that the inflow of 3e strains has occurred several times throughout history, or that several 3e lineages disseminated at some time in the past. Moreover, the Japan-indigenous 3e lineages are clearly nested within the European 3e strains or lineages. These observations strongly suggest that Japan-indigenous 3e lineages originated in Europe. Recently, we reported that the import of a breed of large pigs from Europe since the 1960s may be responsible for the introduction of the 3e strains in Japan (Nakano et al., 2012). The TMRCAs of Japan-indigenous 3e strains calculated in the present study are around 1960, and confirmed this hypothesis.

We analyzed the 12 occurrences of acute hepatitis E cases in Mie prefecture from 2004 to 2011. The Infectious Disease Surveillance Center in Japan announced that the population of Mie prefecture experienced 16 cases of sporadic hepatitis during the same period, indicating that we could study 75% of the cases from Mie prefecture. As described above, some other 3e strains were isolated from areas outside Mie prefecture. However, the persistent occurrence of eight acute sporadic hepatitis cases by closely related 3e strains from 2004 to 2011 was only observed in Mie prefecture. Acute hepatitis cases in Hokkaido in 2001, Miyagi in 2002, and Ehime in 2004 were entirely solitary cases (Inoue et al., 2006a, 2009a; Mizuo et al., 2005). A blood donor who was infected with the 3e strain was the only case in Tochigi prefecture (Fukuda et al., 2007). The present study is the first report of the unexpected persistent occurrence of hepatitis by European-type genotype 3 HEV, subtype 3e, in a country outside of Europe, although autochthonous hepatitis cases by 3e strains have been reported in the United Kingdom (Banks et al., 2004; Dalton et al., 2007; Ijaz et al., 2005), France (Legrand-Abravanel et al., 2009), and Hungary (Reuter et al., 2009). Interestingly, the area in which the 3e strains were prevalent, namely, Mie prefecture, is located far from the most prevalent areas of Japan-indigenous genotype 3a or 3b, or genotype 4 HEV, namely, Hokkaido and the northeastern region of Honshu.

Despite detailed interviews, the eight 3e cases in Mie prefecture had no clear common risk factor of the infection, for instance, travel to Europe or endemic countries, eating boar or swine meat during the same period or in the same location, engaging in pig farming, blood transfusion, and so on. At present, the origin of HEV infection in these eight cases is unknown; however, it is possible that the strains had an epidemiological linkage of which we were unaware. To obtain more information about the cause of the illness, we calculated the TMRCA of the Mie 3e strains. The TMRCA of the Mie strains, including an Ehime strain, was between 1999 and 2004, according to both the strict and relaxed clock models, suggesting that the Mie 3e strains were introduced into Mie prefecture about 10 years ago, and have persisted since then. By investigating the incidence during that time, we may identify an event related to the indigenization of the European-type genotype 3 lineage. The Mie 3e strains might not have been introduced directly from Europe because the phylogenetic trees indicate that the direct ancestor of Mie and Ehime strains is not a European 3e strain. The Mie 3e strains were nested under Miyagi and Tochigi 3e strains in the 301-nt ORF2 tree, suggesting that the Mie stains might have originally come from Europe to another region in Japan.

Our previous study revealed a high prevalence of HEV in Japanese swine, and suggested that swine serve as reservoirs for HEV infection (Takahashi et al., 2003). The food-borne transmission of HEV through ingestion of raw or undercooked meat, including the liver and intestine from infected swine, is one of the most plausible transmission routes (Yazaki et al., 2003). Ingestion of boar or deer meat is also a possible cause of hepatitis E (Bouwknegt et al., 2007; Reuter et al., 2009; Tamada et al., 2004; Tei et al., 2004). However, the acute hepatitis E cases in this study had no history of consumption of meat from wild animals. In Mie prefecture in 2009, there were 66 pig farms and about 120,000 pigs were bred (http://mie.lin.gr.jp/). Eighty percent of the pork meat produced in Mie prefecture is consumed inside Mie prefecture. Although the eight 3e cases in Mie prefecture, with the exception of case 8, had no clear history of eating swine meat including the liver and intestine, pork meat is one of the most popular ingredients of many dishes. People may eat pork meat in restaurants or even at home as precooked food without realizing it. Persistent occurrence of hepatitis E cases by the closely related minor European HEV 3e strains in a limited local area, coupled with the fact that most of the pork meat produced in Mie prefecture is consumed within Mie prefecture, led us to suspect that locally bred swine is a common transmission source of the Mie European-type HEV strain. Investigation of HEV infection of herds of swine of the same region may shed light on the origin of the Mie European strain, although inspection of HEV infection in pig farms is not easy due to the fear of damage caused by rumors. Instead, we are now beginning to investigate the presence of HEV in pig liver and colon sold as food in Mie prefecture, followed by sequence analysis of the HEV RNA.

Most of the patients infected by Mie 3e strains of hepatitis E were middle-aged males, and in fact, this was also found in a nationwide study of 254 hepatitis cases (Abe et al., 2006). In Japan, hepatitis E cases by 3e strains are relatively rare, and the characteristics have not been adequately investigated. The study of HEV infection in Mie prefecture is important not only for determining the origin and transmission route, but also for comparing the clinical features of hepatitis by major strains and rare 3e strains in Japan.

5. Conclusions

From the results of phylogenetic tree analyses, Japan-indigenous genotype 3 strains were divided into two major clusters, namely, 3a and 3b, and one minor cluster, 3e, along with a few unassigned strains. The Japan-indigenous 3e strains were comparatively minor in Japan, and they seemed to be of European origin. We encountered eight sporadic hepatitis E cases by the European-type genotype 3 HEV, subtype 3e, in an unexpected region of Japan, namely, Mie prefecture, from 2004 through 2011. This is the first report of an unanticipated persistent occurrence of hepatitis by the European type HEV. Coalescent analyses indicated that the Mie 3e strains seem to have intruded into Mie prefecture about 10 years ago. Molecular evolutionary analyses traced the history of the indigenization of minor HEV strains into an unexpected region of Japan.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2012.06.002.

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Supplemental figure 1

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CLINICAL STUDIES

New findings regarding the epidemic history and population dynamics of Japan-indigenous genotype 3 hepatitis E virus inferred by molecular evolution

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Keywords
Bayesian skyline plot – coalescent analysis – epidemic history – genotype 3 – hepatitis E virus – Japan – large-race pig – large-scale pig breeding – phylogenetic tree analysis – zoonosis

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Abstract

Background: Since previous studies have investigated the population dynamics of Japan-indigenous genotype 3 hepatitis E virus (HEV) using virus sequences, more nucleotide sequences have been determined, and new techniques have been developed for such analysis. *Aims:* To prevent future hepatitis E epidemic in Japan, this study aimed to elucidate the cause of past HEV expansion. *Methods:* The epidemic history of Japan-indigenous genotype 3 HEV was determined using the coalescent analysis framework. Bayesian skyline plot (BSP) and Bayesian estimate of phylogeny with relaxed molecular clock models were calculated using Markov chain Monte Carlo sampling. *Results:* Japan-indigenous strains consist of New World strains (subtype 3a), Japanese strains (3b) and European strains (3e). The oldest lineage, 3b, appeared around 1929. Lineages 3a and 3e appeared around 1960. BSPs indicated similar radical population growth of the 3a and 3b lineages from 1960 to 1980. *Conclusions:* Population dynamics of the three lineages shared some common characteristics, but had distinguishing features. The appearance of 3a and 3e lineages coincides with the increase of large-race pig importation from Europe and the USA after 1960. The epidemic phase of 3a and 3b strains from 1960 to 1980 could be related to increased opportunity for HEV infection arising from large-scale pig breeding since 1960. Our observations revealed new findings concerning the close relationship between the epidemic history of Japan-indigenous genotype 3 HEV and the improvement of the Japanese pig industry. Infection control in pig farms should be an effective method of preventing HEV infection in humans.

Hepatitis E virus (HEV) is a member of the genus *Hepevirus* in the family *Hepeviridae* (1). HEV is a non-enveloped virus, and its genome comprises positive-sense single-stranded RNA of approximately 7200 nt (2, 3). The genome contains three open reading frames (ORFs). ORF1 codes for non-structural genes, ORF2 encodes the structural protein comprising the virion capsid and ORF3, which mostly overlaps ORF2, encodes a short protein of 113–114 amino acids that is required for virus egress from cells, and is proposed to perturb numerous cellular pathways (4–6).

The GenBank/EMBL/DDBJ accession numbers of the new HEV sequences in this study are AB578953-AB578957, AB578959-578963 and AB581581- AB581598.

Hepatitis E virus is the causative agent of hepatitis E. The first outbreaks of hepatitis E described in the literature were waterborne and associated with faecal contamination of water sources (7, 8). Until its discovery in swine, hepatitis E was assumed to be limited to developing countries and was seen in industrialized countries only as imported cases (7, 9). However, sufficient evidence from Japan and Europe, in the form of sporadic cases who had never travelled to developing countries, suggests that HEV can be transmitted zoonotically to humans from consumption of meat or offal of swine, wild boar and wild deer (10–14).

Hepatitis E virus had been segregated into four genotypes, i.e. 1–4. Lu *et al.* proposed genotyping and subtyping of global HEV strains using phylogenetic analyses (15). Recently, new genotype candidates have been reported in rats (16), rabbits in China (17) and wild boars in Japan (18, 19). The contribution of the new genotype candidates to human disease remains unknown.

Genotypes 1 and 2 do not infect swine (20), infect only humans and are associated with hepatitis E outbreaks by faecally contaminated water sources in developing countries (7–9). Genotypes 3 and 4 have been isolated from sporadic cases in humans who had never travelled to developing countries (21) and from some mammals (13). Genotype 3 strains have been isolated all over the world – Europe, Asia, New World, Oceania and Africa (15, 22). Genotype 4 cases have been reported in Asia, mainly Japan and China (15). Although some cases of genotype 3 and 4 hepatitis have been suggested to occur via zoonotic transmission by exposure to the body fluids of infected swine (23) and ingestion of food products from pigs, boars and deer, the aetiology of other cases with these genotypes still remains to be determined.

In Japan, autochthonous acute hepatitis E accounted for 10–15% of non-ABC hepatitis after 2002 (24). Our previous study revealed a high prevalence of HEV in Japanese swine and suggested that swine serves as reservoirs for HEV infection (25). The food-borne transmission of HEV through ingestion of raw or undercooked meat including liver and intestine from infected swine is one of the most plausible transmission routes (10). A nationwide survey revealed that genotype 3 is the most prevalent genotype in humans (26, 27) and swine in Japan (25). The existence of multiple lineages of genotype 3 HEV has been reported in swine and humans in Japan (25, 28).

The epidemic history of Japan-indigenous HEV strains has already been reported using a coalescent-based method to analyse viral sequences (29). However, only a small number of sequences were previously available for the analyses. Furthermore, the epidemic history was inferred from a single estimated genealogy, and thus the error associated with phylogenetic reconstruction, which may be large, was ignored. Herein, we utilized a more powerful method called the Bayesian skyline plot (BSP) (30, 31), which is not influenced by phylogenetic error. Moreover, a more realistic Bayesian inference method with relaxed clock models that incorporate variation in evolutionary rates among lineages has been developed (32). Purdy and Khudyakov reported an epidemic history of all genotypes of HEV strains using this method (33).

In this study, we focused on the epidemic history of Japan-indigenous genotype 3 HEV and analysed the sequence of Japanese strains on a large scale using the sophisticated Bayesian inference method. Comprehensive phylogenetic analyses comparing Japanese genotype 3 strains and genotype 3 strains from other countries were also performed, and reconfirmed the existence of multiple lineages in Japan. New interesting findings on the epidemic history and past population dynamics of Japan-indigenous genotype 3 HEV were revealed using molecular evolutionary analyses considering multiple lineages. We also speculate on a possible dissemination scenario for Japan-indigenous genotype 3 HEV, which could be applied to the prevention of future endemic or epidemic HEV infection in Japan and other developed countries.

Materials and methods

Sampling, isolation, and sequencing of HEV isolated from 2009–2010 HEV infections in Japan

Nine genotype 3 HEV strains isolated from patients admitted to Toshiba General Hospital and Tokyo Teishin Hospital, Tokyo, Japan in 2009 and 2010 were included. The patients had undergone an interview for risk factors pertaining to HEV infection and represented sporadic cases that were not correlated to each other. Informed consent was obtained from each patient. This study was approved by the institutional review committees of Toshiba General Hospital and Tokyo Teishin Hospital.

A total of 313 packages of raw pig liver and colon sold as food were purchased from 22 grocery stores in Tokyo in 2009. Tissue specimens were obtained from each package. Eight pig liver or colon specimens had detectable HEV RNA (34). The nucleotide sequences from the patients and pig livers or colon were determined using a previously reported method (35), with some modifications. In brief, nucleic acids were extracted from serum with the QIAamp MinElute Virus Spin Kit (QIAGEN GmbH, Hilden, Germany). HEV RNA genomes were reverse transcribed, and cDNA was amplified using PCR with primers specific for targeted partial ORF1 and ORF2 regions of the HEV genome (28, 36). Reverse transcription and first-round PCR were conducted using the SuperScript III One-Step RT-PCR System (Invitrogen Corporation, Carlsbad, CA, USA); second-round PCR was conducted using Platinum Taq DNA polymerase (Invitrogen). The final products were sequenced in the 377 DNA Sequencer using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). For five

swine and nine human strains, both the ORF2 and ORF1 region sequences were determined in this study, and these strains were included in the subsequent phylogenetic and coalescent analyses. Only one region of the ORF2 or ORF1 sequence was successfully sequenced for the remaining three swine strains; therefore, those three strains were not included.

Phylogenetic analysis of the ORF2 and ORF1 datasets

All available nucleotide sequences of 301 nt of ORF2 (nucleotide number 5994–6294: M73218) and 287 nt of ORF1 (nucleotide number 104–390) were downloaded from the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp/index.html) (37). These two regions have been sequenced and frequently used for genotyping and subtyping since Mizuo *et al.* compared Japanese HEV strains with global HEV sequences (28). Lu *et al.* reviewed genotyping and subtyping of global HEV strains by phylogenetic analyses using these regions (15). Database sequences were divided into each genotype using phylogenetic analyses after comparison with reference sequences for each genotype: genotype 1, M73218; genotype 2, M74506; genotype 3, AB073912; genotype 4, AB074915; Chinese rabbit HEV, FJ906895 (17); genotype 5, AB576193 (18); and newly reported novel genotype, AB602441 (19).

Using SeaView version 4 (38), large trees were estimated using neighbour-joining (Kimura 2-parameter substitution model). Only genotype 3 sequences and the above reference sequences were retained for the analyses. Next, information from the database, from original publications and from our own knowledge was used to classify the sequences. Only one sequence from each infected individual was retained. Closely related sequences obtained from single outbreaks of hepatitis E from the same origin were excluded except for one sequence that was considered as the representative. Similar sequences isolated from the same swine herd were also excluded except for one sequence. Experimental clones were excluded. Sequences whose nationality remained unknown were also excluded. However, identical or very similar sequences whose epidemiological linkage was not certified by any means could not be excluded because of the absence of a rational exclusion criterion for random sampling.

Database sequences were then collated and aligned with our 14 newly generated Japanese genotype 3 sequences, and were subsequently adjusted manually. The resulting ORF2 and ORF1 alignment contained 473 and 131 global genotype 3 sequences respectively (Tables 1 and 2). Neighbour-joining trees and maximum likelihood (ML) phylogenies of the two datasets were constructed to determine the phylogenetic distribution of the included Japanese strains within the global epidemic using SeaView version 4. The program jModelTest was used to select the most appropriate nucleotide substitution model that adequately fit the sequence datasets (39). Maximum likelihood phylogenies were heuristically searched using the SPR (subtree pruning and regrafting) and NNI (nearest neighbour interchange) perturbation algorithms. The statistical robustness levels of phylogenetic groupings were subsequently assessed using bootstrap analyses (1000 replicates for neighbour-joining trees and 100 replicates for ML phylogenies). Phylogeographic structure was then identified using FigTree (available from http://tree.bio.ed.ac.uk), and ancestral lineage states of clades and lineages of Japanese strains were coloured using a parsimony approach (40). Colours were used according to the subtyping, tentatively determined by Lu *et al.* (15).

Table 1. Origin of and number of isolates included in each phylogeny cluster of the 301-nt ORF2 region

3a		3e	
Japan	59*	Japan	17
Korea	24	Mongolia	1
Thailand	5	UK	16
China	1	France	5
Taiwan	1	Hungary	2
USA	28	Germany	1
Canada	20	Russia	1
Mexico	5	total	43
Hungary	6		
Netherland	2	3f	
Total	151	Japan	2
		Thailand	5
3b		France	63
Japan	137†	Spain	14
China	4	Netherland	7
Canada	1	Germany	2
France	1	UK	2
Total	143	Sweden	1
		Total	96
3c			
Mongolia	1	3g	
France	10	Kyrgyzstan	1
Netherland	4	Total	1
Germany	3		
Brazil	4	3j	
Congo	1	Canada	5
Total	23	Total	5
		Magenta (undetermined)	
3d		Japan	6
Taiwan	3		
Total	3	Cyan (undetermined)	
		Japan	2

*Two newly determined isolates in this study are included.
†Twelve newly determined isolates in this study are included.

Coalescent analyses of Japan-indigenous genotype 3 HEV strains

From the genotype 3 Japanese ORF2-301 nt dataset and ORF1-287 nt dataset, sequences of imported cases were

Table 2. Origin of and number of isolates included in each phylogeny cluster of the 287-nt ORF1 region

3a		3f	
Japan	22*	Japan	2
Korea	2	Thailand	2
USA	3	Mongolia	1
Hungary	4	Netherland	10
Germany	2	Spain	6
Netherland	2	France	1
Total	35	Germany	1
		Greece	1
3b		Sweden	1
Japan	68†	Total	25
China	1		
Total	69‡	3g	
		Kyrgyzstan	1
3c		Total	1
Netherland	5		
Germany	1	3h	
total	6	Mongolia	1
		Itay	1
3e		NewZealand	1
Japan	6	Total	3
UK	1		
Hungary	1	3i	
Germany	1	Argentina	3
Greece	1	Germany	1
Total	10	Austria	1
		Total	5
		3j	
		Canada	1
		Mexico	1
		Australia	1
		Total	3

*Two newly determined isolates in this study are included.
†Twelve newly determined isolates in this study are included.
‡One undetermined isolate in ORF2 phylogeny is included.

excluded, and human, swine and wild animal sequences that strongly suggested domestic infection within Japan were retained. Many of the sequences with known sampling dates were longer sequences including the 301-nt ORF2 region or 287-nt ORF1 region. Sampling dates of each sequence were accurate because most dated sequences were obtained by this study's authors. Including 14 newly generated sequences in this study, 174 Japan-indigenous ORF2 sequences (412 nt: nucleotide number 5944–6355) and 76 ORF1 sequences (326 nt: nucleotide number 125–450) were available as sequences with known sampling dates. The sampling dates of the longer ORF2 dataset and the longer ORF1 dataset ranged from 9 October 1979 to 23 June 2010 and from 21 September 1993 to 23 June 2010 respectively. Furthermore, a concatenated dataset was also analysed to increase statistical power, but at the expense of a reduction in the number of available strains for analyses. The concatenated dataset contained 59 Japan-indigenous sequences.

From the dated sequence datasets, the evolutionary rate and epidemic history of Japan-indigenous genotype 3 HEV were inferred using the framework of coalescent analysis implemented in BEAST (41). Markov chain Monte Carlo (MCMC) sampling was performed for at least 1×10^8 generations, sampling a tree for every 10 000 generations. The general time-reversible model with rate heterogeneity among sites and invariable sites (GTR + Γ + I) model was selected for the dated sequence sets using jModelTest from among 24 models. To select the best-fitting molecular clock from among a strict molecular clock and relaxed molecular clocks (uncorrelated lognormal and uncorrelated exponential), Bayes factors (BFs) were estimated for each dataset in a Bayesian framework using the Tracer program (http://tree.bio.ed.ac.uk). The Tracer was used to check for convergence and to determine whether appropriate mixing of the posterior target distribution had been achieved (effective sample size > 200). Tracer was also used to reconstruct BSPs to show the epidemic history of Japan-indigenous genotype 3 HEV. The BSP is a combined plot of variants of the generalized skyline plots determined by generating a posterior distribution of effective population size through time using MCMC sampling procedure, given the sequence data (31).

A Bayesian estimate of phylogeny was obtained from the posterior distribution of trees arising from the best-fitting BEAST analysis. Firstly, the program, TreeAnnotator (41), was used to construct a phylogeny that best summarizes the set of credible trees and is called the maximum clade support phylogeny. As a relaxed clock was used in the Bayesian MCMC analysis, the branch lengths and node heights of the maximum clade support phylogeny are in units of years (32). Phylogeographic structure was then identified using FigTree, and clades and lineages were coloured in the same manner as the trees of shorter sequences.

Results

Phylogenetic analyses of the global ORF2 and ORF1 datasets

In the neighbour-joining tree of a 301-nt sequence within the ORF2 region, subtypes of clusters were guided by subtyping tentatively determined by Lu *et al.* (15) (Fig. 1). According to the increasing number of sequences, the subtyping of the clusters became ambiguous, and no significant bootstrap support was obtained for each subtype. In the ML tree, no significant bootstrap support was obtained for each subtype (data not shown). In spite of the ambiguity of the subtyping in the large phylogeny of the short sequences, the structure was generally consistent with that of the small phylogeny based on corresponding full genome sequences (data not shown).

Then, 473 genotype 3 strains were tentatively divided into 3a, 3b, 3c, 3d, 3e, 3f, 3g, 3j and undetermined subtypes respectively (Fig. 1). Each cluster contained different ratios of Asian, European, New World and African

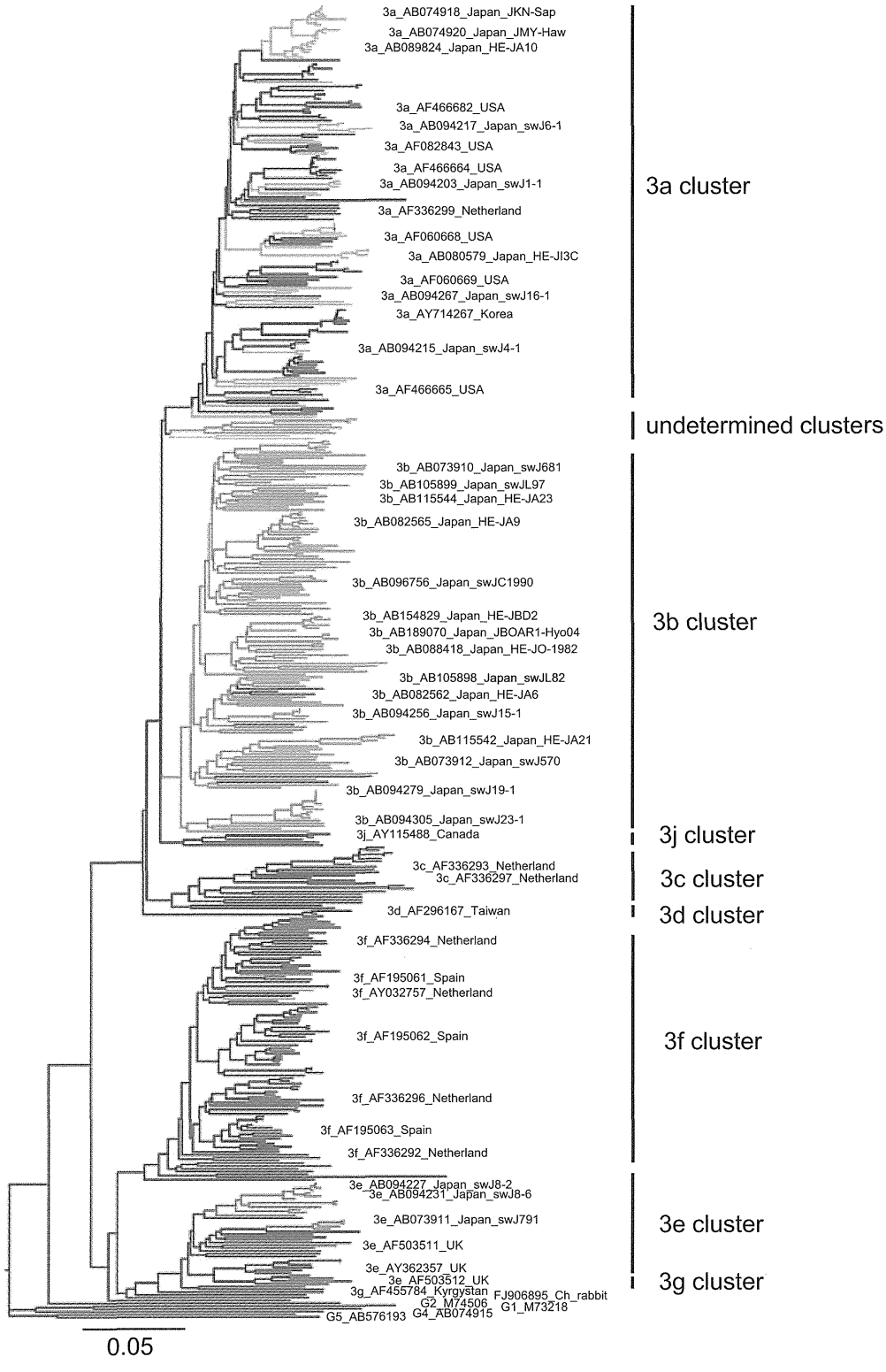

Fig. 1. Neighbour-joining tree of 301-nt sequence alignment within the ORF2 region containing 473 genotype 3 strains and outgroup strains. As the large size of the tree arising from the high number of sequences makes indicating all strain names difficult, only sequences reported by Lu *et al.* are indicated as guides for subtyping (15). The identical tree showing all strain names is available upon request. The ancestral lineage states of clades are indicated using a parsimony approach. The lineages of Japanese strains are coloured: 3a, green; 3b, red; 3e, blue; 3f, brown; and undetermined lineages, magenta and cyan. The lineages from other countries remain black.

strains, as shown in detail in Table 1. Overall, the 3a cluster consisted of many groups of Asian strains including Japanese strains and many groups of New World strains. The 3b cluster consisted mostly of Japanese strains. The 3c cluster consisted chiefly of European and some other strains. The 3e cluster consisted of two groups of 17 Japanese strains and many European strains. The 3f cluster consisted of mostly European strains. Two Japanese strains were included in this cluster, but these strains were strongly suggested as imported cases (42) (published only online). European strains were included in most of the subtypes. As a result, Japan-indigenous strains were divided into many groups: 3a strains that related to New World strains, 3b strains that consisted mostly of Japanese strains, 3e strains that were related to European (mainly UK) strains and unassigned strains.

In the neighbour-joining tree of the 287-nt sequence in the ORF1 region, subtyping by Lu *et al.* also served as a provisional standard (Fig. 2). In this tree, the structure of the subtyping also became ambiguous, and no significant bootstrap support was obtained for each subtype. In the ML tree, no significant bootstrap support was obtained for each subtype (data not shown). However, the structure was generally consistent with that of the above-mentioned ORF2 phylogeny. In the ORF1 tree, strains were divided into 3a, 3b, 3c, 3e, 3f, 3g, 3h, 3i and 3j clusters. The detailed composition of the countries of origin of each strain in each subtype is shown in Table 2. Overall distribution of Asian, European and New World strains in each cluster were the same as the ORF2 tree. The 3d cluster did not exist, and instead, 3h and 3i clusters were observed. Two Japanese 3f strains were the same strains as those in the ORF2 tree, that is, imported cases. As a result, Japan-indigenous strains were divided among 3a strains, 3b strains and 3e strains as in the ORF2 tree. One Japanese strain (Magenta) that was unassigned in the ORF2 tree was included in cluster 3b, probably because of the weak phylogenetic signal of the short region.

Two newly determined Japanese sequences in this study were included in cluster 3a, and 12 were included in cluster 3b in both ORF2 and ORF1 phylogenies. From the results of the ORF2 and ORF1 region trees, the Japanese strains in the 3a, 3b, 3e and undetermined clusters were analysed as Japan-indigenous strains in the following analyses.

Estimation of epidemic history and population dynamics of Japan-indigenous genotype 3 HEV using dated ORF2 dataset

Evolutionary analysis of the dated ORF2 dataset was performed with a strict molecular clock and a relaxed molecular clocks (uncorrelated lognormal and uncorrelated exponential) in BEAST. The combination of the three clock models and BSP gave similar median estimates of the evolutionary rate and time of the most recent common ancestors (TMRCAs) of the total tree and each clade (Table 3). Although the strict clock model gave a narrow range of evolutionary rate, the uncorrelated exponential clock model gave the best BF (vs. strict clock: 11, vs. uncorrelated lognormal clock: 10) and also gave the widest range of evolutionary rate and TMRCAs. The evolutionary rate was 1.44×10^{-3} (95% credible interval (CI), 0.95×10^{-3} to 1.99×10^{-3}) substitutions/site/year. The TMRCAs dated 1847 (1675–1940) for the total tree, 1959 (1940–1974) for cluster 3a, 1929 (1873–1961) for cluster 3b and 1960 (1937–1974) for cluster 3e respectively.

Figure 3 shows the maximum clade support phylogeny for the dated ORF2 sequence set, reconstructed from the phylogenies sampled under the best-supported model combination: the uncorrelated exponential clock model and BSP. Japan-indigenous 3a, 3b, 3e and undetermined cluster strains each clustered together. The tree demonstrated a similar structure to the global ORF2 region tree. The 3e cluster branched off from other clusters at the earliest time (around 1850), without more branching until after 1950. The 3b cluster, which consisted mostly of Japanese strains, began to branch between 1900 and 1950 followed by more frequent branching after 1950. The 3a cluster and the undetermined clusters also started to branch after 1950.

Figure 4a shows the BSPs estimated from the Japan-indigenous 3a and 3b clusters of the ORF2 dataset. The BSP is a flexible, non-parametric estimate of past changes in effective population size (number of infections). The most notable difference in BSPs between clusters 3a and 3b was the different time of onset. The BSP of cluster 3a began around 1960, but the BSP of cluster 3b began around 1930. The same difference between the two clusters was seen in TMRCAs (Table 3) and the beginning of branching (Fig. 3). The effective population size of 3a rapidly increased from around 1960. The population size of 3b demonstrated a relatively constant phase from 1930 to 1960, and then indicated rapid epidemic growth. This rapid growth of the two BSPs coincided with the onset of rapid diversification in the lineages of the two clusters after 1960, as shown in Fig. 3. The rate of growth appeared to slow around 1980, and the population size entered a constant phase until around 2000 in both BSPs. From 2000 to the present, the population size decreased in both BSPs. The number of 3e strain sequences was too small to be analysed by BSP.

Estimation of epidemic history and population dynamics of Japan-indigenous genotype 3 HEV using a dated ORF1 dataset and concatenated dataset

To support the results of the ORF2 dataset, evolutionary analyses of the dated ORF1 dataset and the dated concatenated dataset were performed in BEAST by the same methods used for the dated ORF2 dataset. The uncorrelated exponential model gave the best BF among the

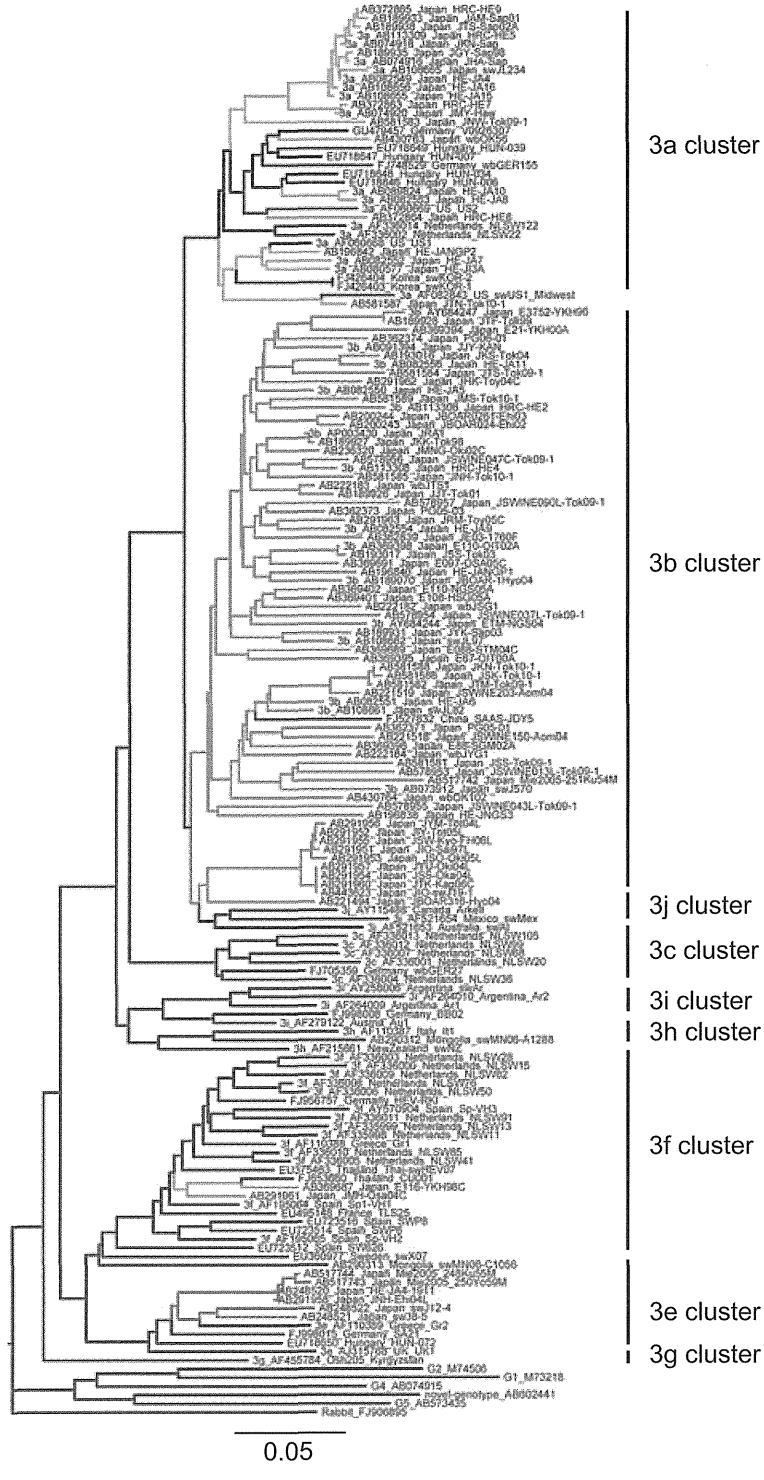

Fig. 2. Neighbour-joining tree of 287-nt sequence alignment within the ORF1 region containing 131 genotype 3 strains and outgroup strains. The ancestral lineage states of clades are indicated using a parsimony approach, and colours have the same indications as in Fig. 1. Strain subtypes reported by Lu *et al.* are indicated as guides for subtyping at the beginning of strain names. The origin of each strain is also indicated in strain names.

Table 3. Clock rates and the time of the most recent common ancestors (TMRCAs) (median and 95% credible intervals)

		Relaxed uncorrrelated clock	
Clock	Strict clock	Lognormal	Exponential
ORF2			
Clock rate*	1.35 (1.01–1.69) × 10^{-3}	1.35 (1.01–1.70) × 10^{-3}	1.44 (0.95–1.99) × 10^{-3}
TMRCAs			
Total	1804 (1719–1871)	1805 (1714–1875)	1847 (1675–1940)
3a+3b	1925 (1904–1942)	1924 (1903–1942)	1914 (1846–1953)
3a	1961 (1950–1969)	1961 (1949–1969)	1959 (1940–1974)
3b	1943 (1927–1955)	1943 (1926–1956)	1929 (1873–1961)
3e	1958 (1942–1968)	1959 (1943–1969)	1960 (1937–1974)
ORF1			
Clock rate*	1.10 (0.35–1.93) × 10^{-3}	1.09 (0.32–1.95) × 10^{-3}	1.47 (0.04–3.42) × 10^{-3}
TMRCAs			
Total	1661 (1181–1871)	1655 (1114–1876)	1762 (893–1968)
3a+3b	1891 (1747–1954)	1888 (1722–1954)	1882 (1468–1979)
3a	1936 (1849–1973)	1935 (1836–1974)	1943 (1760–1989)
3b	1918 (1808–1965)	1916 (1791–1966)	1923 (1648–1987)
3e	1940 (1861–1974)	1939 (1850–1974)	1953 (1797–1991)
Concatenated set			
Clock rate*	1.19 (0.46–1.90) × 10^{-3}	1.07 (0.32–1.82) × 10^{-3}	1.32 (0.09–3.32) × 10^{-3}
TMRCAs			
Total	1698 (1367–1855)	1664 (1191–1864)	1749 (892–1969)
3a+3b	1896 (1783–1946)	1882 (1721–1947)	1870 (1462–1978)
3a	1947 (1888–1974)	1940 (1855–1974)	1941 (1760–1989)
3b	1925 (1840–1961)	1915 (1797–1962)	1920 (1681–1987)
3e	1942 (1883–1971)	1937 (1852–1970)	1951 (1798–1990)

*Substitutions/site/year.

three clock models, but it was not significant, possibly because of insufficient information from the small number of sequences. As a result, the estimates of evolutionary rate and TMRCAs under the uncorrelated exponential model had considerably wide 95% CI (Table 3). However, the median estimates were not very different from those in the ORF2 region analysis.

Figure 5a, b show the maximum clade support phylogenies for the dated ORF1 sequence set and the dated concatenated dataset. Japan-indigenous 3a, 3b, 3e and undetermined strains clustered together. The trees showed structure similar to the ORF2 region maximum clade support phylogeny. The 3e strains branched at the oldest age without more branching until 1950. The 3b cluster began to branch between 1900 and 1950, with branching frequently after 1950. The 3a cluster started to branch after 1950.

Figure 4b, c show the BSPs estimated from the Japan-indigenous 3a and 3b clusters of the dated ORF1 dataset and the dated concatenated dataset. The BSPs of the two clusters had a wide range of 95% highest posterior density confidence intervals, corresponding to ambiguous estimates of the evolutionary rate and wide range of TMRCAs. However, the median estimates of the four BSPs represented an increase around 1960, constant phase from 1980 to 2000 and decrease in effective population size after 2000, which were all seen in the ORF2 BSPs.

Discussion

In a report on the epidemic history of Japan-indigenous genotype 3 HEV strains by Tanaka *et al.* (29), the identity of the Japan-indigenous strains could not be investigated sufficiently, and differences in epidemic histories by lineage could not be considered adequately because of the small number of analysed sequences. In this study, we first tried to define the Japan-indigenous genotype 3 HEV strains compared with the global strains. Most sequences reported from various countries are relatively short in the ORF2 and ORF1 regions, although analysis of longer or even whole-genome sequences should be more reliable for accurate identification of genetic relatedness among HEV strains. Especially for European strains, longer or full genome sequences were so limited that analysing two short regions was unavoidable for the identification of Japan-indigenous strains compared with the global strains. The more the sequences are added, the more ambiguous the subtyping becomes, and clusters for each subtype are not supported using bootstrap analyses in neighbour-joining trees and ML trees. However, the structure of the trees of short sequences is almost the same as that of the full genome sequence tree (data not shown).

In this study, three major lineages of Japan-indigenous strains were discerned, clusters 3a, 3b and 3e, according to the tentative subtyping by Lu *et al.* (15).

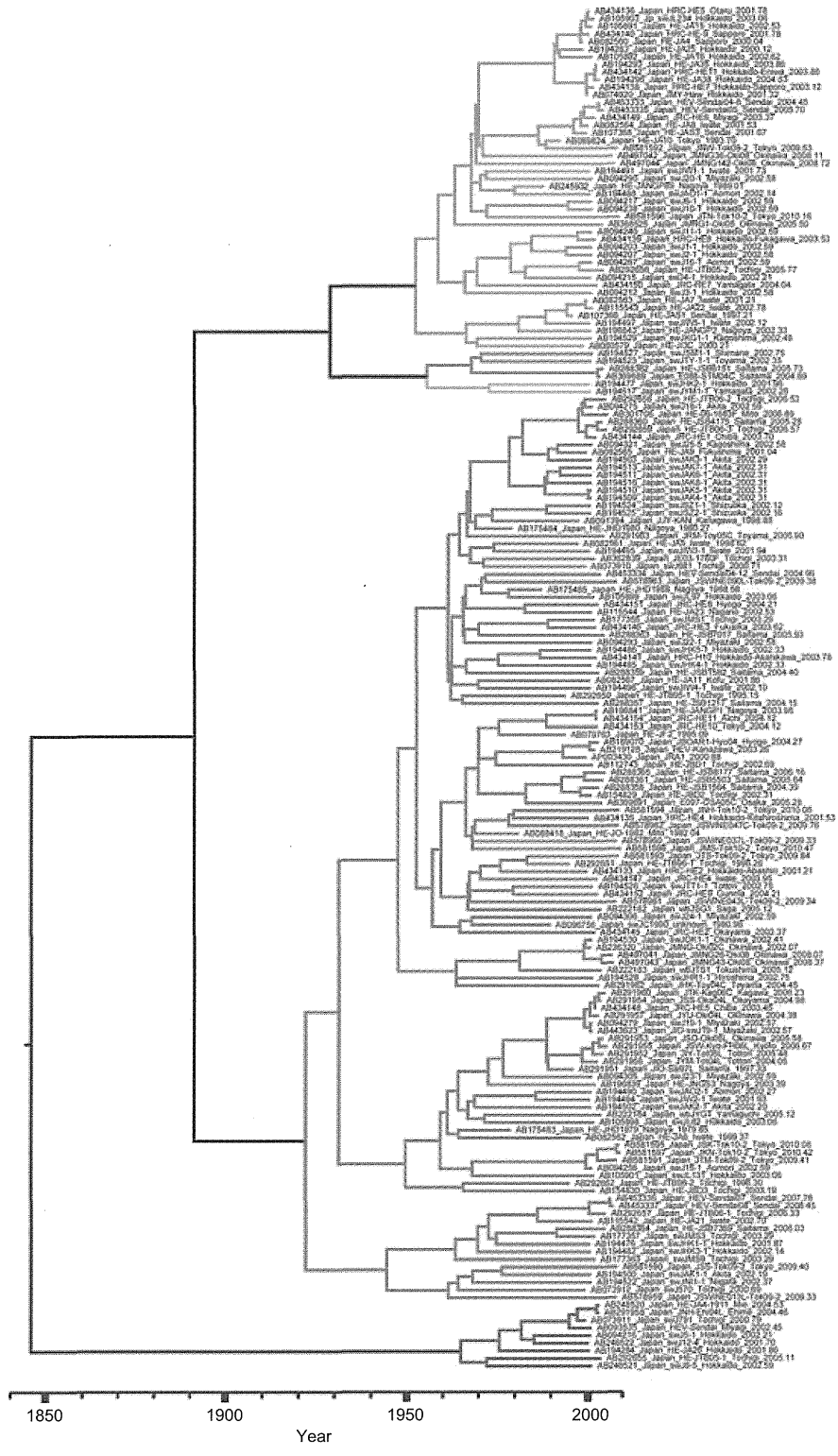

Fig. 3. Maximum clade support phylogeny for the dated Japanese sequence set of the ORF2 region (412 nt). Japan-indigenous 3a (green), 3b (red), 3e (blue) and undetermined (magenta and cyan) clusters indicate a structure similar to the global ORF2 region tree. The branch lengths and node heights are in units of years.

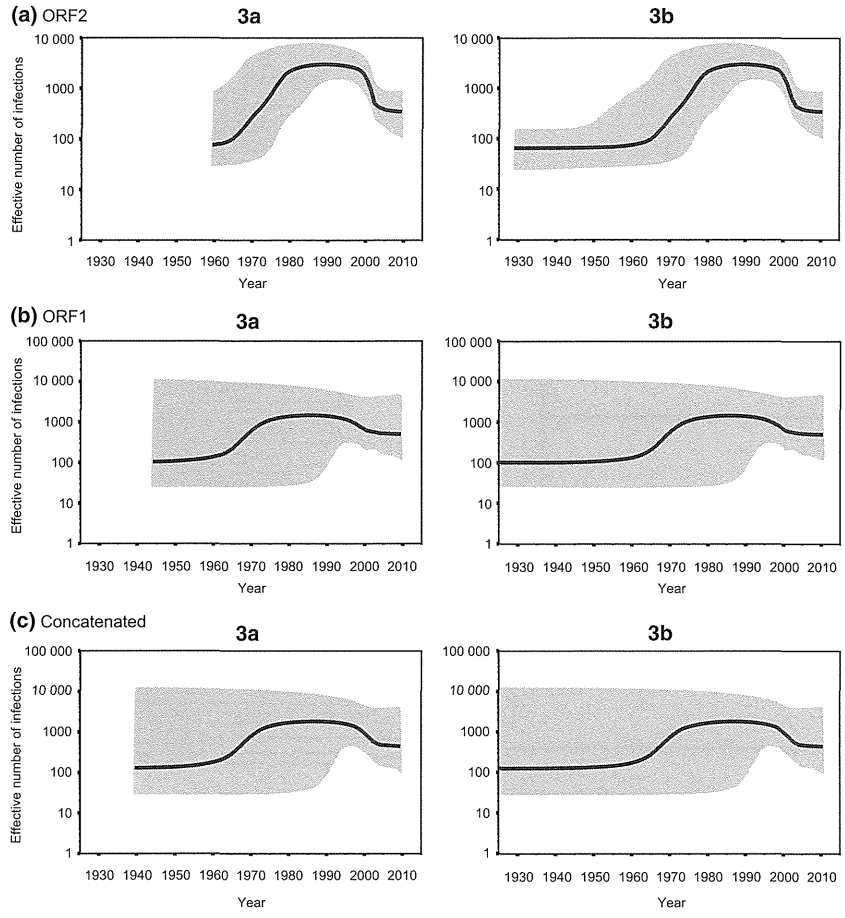

Fig. 4. Bayesian Skyline plots estimated from the Japan-indigenous 3a and 3b clusters of the ORF2 dataset (a), ORF1 dataset (b) and concatenated dataset (c). The thick black line represents the estimated effective population size through time. The grey area represents the 95% highest posterior density confidence intervals for these estimates.

The relationships among the three major lineages and strains from various countries shown in Figs 1 and 2 suggest that epidemic history may differ among the three lineages, and their histories should be studied separately. Other minor lineages, such as the undetermined clusters in Figs 1 and 3, may also be indigenous. Consideration of the different epidemic histories of the three lineages led to the detailed population dynamics of Japan-indigenous genotype 3 HEV infection, which could not be observed in the study of Tanaka *et al.* (29).

The 3b cluster consists mostly of Japanese strains, with the highest number of isolated strains among the three major Japanese genotype 3 lineages. The 3b cluster is probably the major type of Japan-indigenous genotype 3 HEV. However, its origin or specific relationship with strains from other countries is not distinguished by the structure of the trees constructed by the short ORF2 or ORF1 region. In the 3a cluster, many groups of Japanese strains are intricately intertwined with many groups of strains from New World countries. However, no individual Japanese strain demonstrated a direct close relationship with strains from individual New World countries. The presence of multiple old phylogenetic nodes that connect Japanese clusters and lineages from New World countries (Figs 1 and 2) suggests that historical inflow of 3a strains occurred several times, or several 3a lineages disseminated at some time in the past. The direction or origin of the inflow or dissemination is not clear from the short region trees. The relatively minor lineage, Japanese cluster 3e, is clearly nested within the European 3e cluster, which consists mainly of UK strains. This observation strongly suggests that Japanese 3e lineage originated in Europe, most likely in the UK.

Recent molecular and serological data have led to the consensus that hepatitis E arising from genotype 3 and 4 strains involves zoonosis with a reservoir in pigs and possibly a range of other mammals (10, 12, 14, 43). Many of the HEV infections in Japan are most likely zoonotic in origin (8). Particularly, swine-borne infection is a major route of HEV infection in Japan (10, 44, 45). Although genotype 3 HEV infections in wild boars or other mammals have been reported in Japan (12, 14,

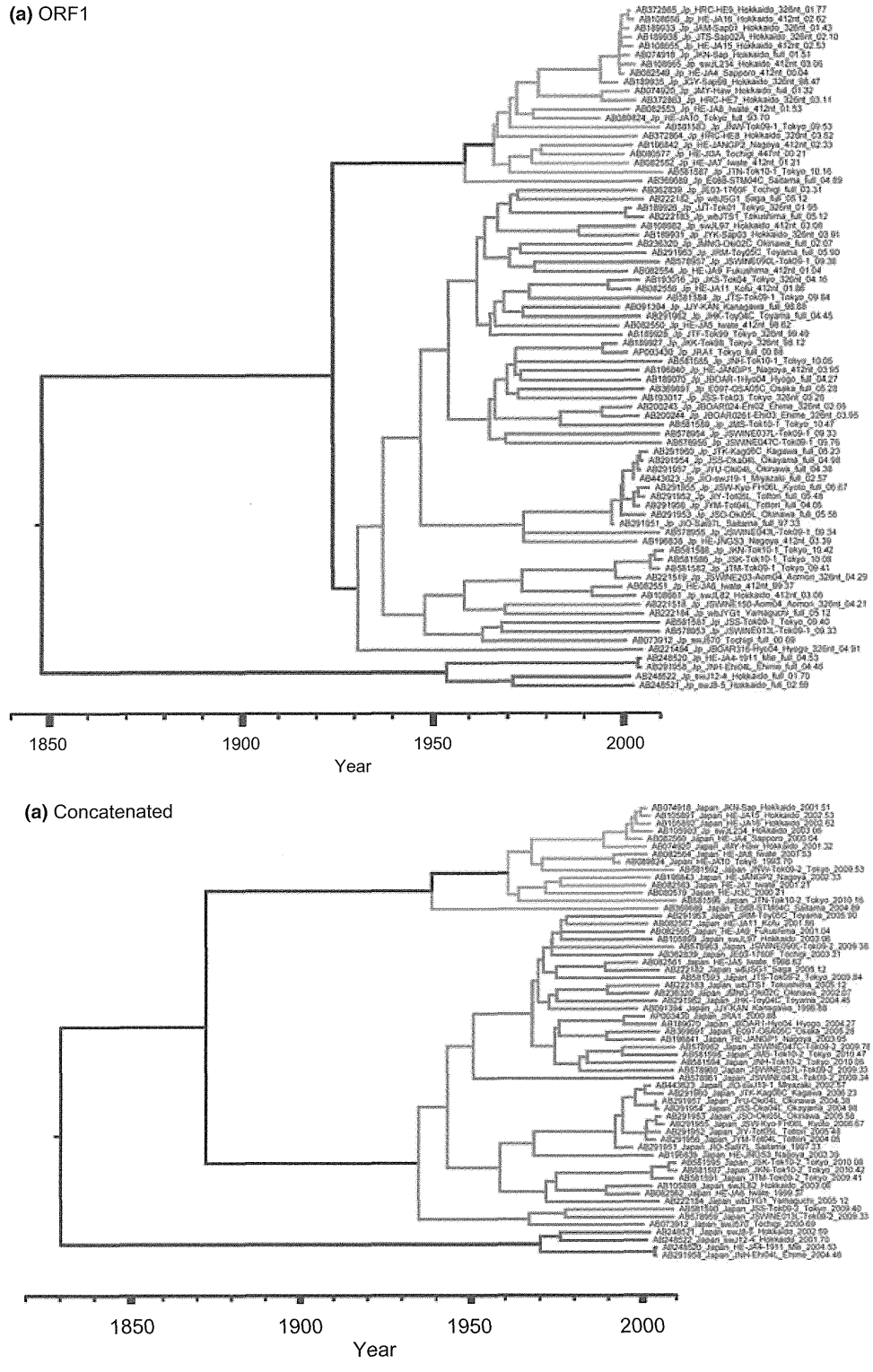

Fig. 5. Maximum clade support phylogeny for the dated Japanese sequence set of the ORF1 region (326 nt) (a) and concatenated dataset (b). The lineages are coloured as in the ORF2 tree (Fig. 1). The branch lengths and node heights are in units of years.

46), their impact on the total number of genotype 3 infections may be limited.

Until the modern age, eating pork was traditionally inhibited by Buddhism in Japan. Japanese governments then changed their policy to ensure that the people (especially soldiers) gained strength by eating meat, and have encouraged the pig industry since the 1870s. The history of the Japanese pig industry has been recorded in detail in Japanese (http://www.jppa.biz/book.html). The Japanese government began importing pedigree pigs in 1869 to improve the pig industry. The Japanese government imported 270 pigs (Berkshire, Yorkshire, and others) between 1899 and 1936, mostly from the UK. Some private companies also imported 137 pigs between 1923 and 1938 from the UK and USA. Other government branches also imported Berkshire, Yorkshire and other breeds of pigs from the UK, USA and Canada until the beginning of World War II.

This period is almost concordant with our TMRCA results for cluster 3b (Table 3). However, small-scale breeding, with 1 or 2 pigs per pig breeder, as conducted until 1960, could not influence a drastic population change of swine HEV infections, and may then represent an endemic constant phase of infections until 1960 in spite of frequent importation of many pigs. It seems that one limited lineage, 3b, which entered Japan from Western countries before World War II, established the major lineage in Japan. Most pigs were imported from the UK, but cluster 3b was nested within the 3j cluster, which included Canadian strains in the ORF2 phylogeny. Although Tanaka *et al.* previously suggested that Japan-indigenous genotype 3 was imported from the UK by quoting a report indicating a close phylogenetic relationship between two UK genotype 3 swine strains and some Japanese swine strains (29, 47), the UK and Japanese strains belonged to subtype 3e, and not 3b. The precise origin of the major lineage of Japan-indigenous genotype 3 strains, cluster 3b, will remain unclear until more reliable phylogentic analyses using a sufficient number of longer or even whole-genome nucleotide sequences from other countries' genotype 3 strains are performed.

According to the historical records, the Japanese pig industry developed gradually until World War II (1939–1945), but faced extinction because of difficulty in obtaining feedstuff during and after the War. Our results of coalescent analyses show two new findings after 1960: the appearance of two other lineages, 3a and 3e, and similar radical population growth of the lineages until 1980. These two big changes can also be explained by two improvements that occurred in the modern Japanese pig industry after 1960. One of these improvements is the beginning of the breeding of large pigs. After 1960, Japanese pig breeders imported large-race pigs to increase economic efficiency. Several types of large pigs, i.e. Landrace, large Yorkshire, Hampshire and Duroc, were imported from the UK, the Netherlands, Sweden, Denmark and the USA. Importation of large pigs from several countries may be a possible reason for the introduction of the 3a and 3e HEV strains and the multiplicity of the 3a lineages.

The other improvement is the increase in the number of pigs at individual pig farms after approximately 1960. Large-scale pig breeding probably increased the opportunity for HEV infections through contact exposure compared with small-scale breeding. Indeed, we reported that almost all 5- or 6-month-old pigs from 25 swine herds throughout Japan had detectable anti-HEV (25). The total number of pigs in Japan was the lowest in 1946, at only 88 000, and began to increase because of large-scale pig breeding from around 1960, reaching approximately 10 million pigs in 1981. Subsequently, the number has remained almost constant to the present. Most of the observations in our coalescent analyses, the endemic phase of cluster 3b, appearance of 3a and 3e lineages, epidemic phase of clusters 3b and 3a from 1960 to 1980 and constant phase after 1980, are concordant with the history of the Japanese pig industry as described above.

The authenticity of the observed decrease of HEV infections in the BSPs of clusters 3b and 3a after 2000 remains unclear. Our previous study conducted between 2000 and 2002 showed that almost all pigs in 25 swine herds throughout Japan had IgG class anti-HEV antibody (25), suggesting that almost all Japanese pigs bred around 2000 were infected by HEV. Since 2002, HEV infection in Japanese pigs based on IgG class antibody has not been studied. As large-scale pig breeding has become common, current pig breeders have come to pay more attention to pig health and sanitation to prevent swine-specific diseases including toxoplasmosis, dysentery, Aujeszky's disease, mycoplasmal pneumonia and atrophic rhinitis. These procedures may also have decreased the chance of pigs getting infected with HEV.

Another possibility is that the decrease observed in the BSPs is an artefact of the sequences used for the analyses. We carefully removed closely related sequences within single outbreaks of hepatitis E from the same origin, leaving just one sequence as the representative. However, identical or very similar sequences whose epidemiological linkage was not certified remained in the datasets. As a result, some identical or very similar sequences were included in our coalescent analyses. Such similar sequences may have had an unknown epidemiological linkage. In other words, close relatedness of sequences may be evidence of close epidemiological linkage. When identical or very similar sequences were removed, and the same analyses were repeated, the decrease in the BSPs after 2000 diminished considerably and was not statistically significant (data available upon request). Therefore, the decrease may be an artefact related to non-random sampling of analysed sequences. It cannot be determined whether the decrease in HEV infection observed in the BSPs after 2000 is true or artefactual until epidemiological reinvestigation of HEV infections in swine herds is conducted throughout Japan.

Recently, Purdy and Khudyakov reported the epidemic history of all genotypes of HEV strains using the same method, but using a different genomic region from that used in the present study (33). In their analyses, they divided genotype 3 HEV into two lineages. One of them (tentatively assigned as lineage 3.1) consisted mainly of Japanese 3b, Japanese 3a and several 3c strains from Germany. The other lineage (tentatively assigned as lineage 3.2) consisted of a few 3e and 3f strains. They observed an endemic phase, epidemic phase, constant phase and decrease to the present of HEV infections in their BSP of lineage 3.1. The shape is similar to our BSP of Japan-indigenous 3b strains analysed from a different genomic region, which supports the reliability of both studies to some extent.

In the present study, we focused on the epidemic history of Japan-indigenous genotype 3 HEV. Our BSP of clusters 3b and 3a indicated an epidemic phase between 1960 and 1980, whereas the BSP of clade 3.1 from the previous study demonstrated an epidemic phase between 1940 and 1960. Increase of HEV infections in this period in Japan is not plausible because the number of pigs serving as an HEV reservoir was the lowest in Japan in those days during and just after World War II. Their analyses are precise and sophisticated, but concerning Japan-indigenous lineages, we analysed many more strains and obtained results supported by historical records. It is possible that sampling error, a smaller number of dated sequences and ambiguous sampling dates may underlie the deviation in timing of the epidemic phase in their analyses.

We attempted to stabilize the molecular clock by adding more information, that is, by including 14 newly determined sequences from 2009 and 2010. The selected molecular clock was not a strict clock model, but a relaxed clock model. The relaxed clock on the evolution of HEV seems realistic. The relaxation may be an innate characteristic of HEV infection, which does not persist chronically in one individual except in particular cases under conditions of immune suppression (48). HEV maintains its population in the community by hopping from host to host. On some occasions, such as under the conditions of swine herds, HEV may infect frequently and simultaneously, followed by rapid evolution. In other situations, as in environments such as sewage, HEV may stop replication and await the opportunity to infect the next host (49, 50), followed by sluggish evolution.

We observed new findings on the epidemic history and population dynamics of Japan-indigenous genotype 3 HEV infections and confirmed the strong relationship between the infection and pig farming. In particular, importation of large-race pigs and the transition to large-scale pig breeding may have considerably influenced the population dynamics of HEV infection. Infection control on pig farms should be the primary effective method for prevention of future endemic or epidemic HEV infection of humans in Japan and other developed countries.

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Case Report

Icteric acute hepatitis E with no response of immunoglobulin M class anti-hepatitis E virus antibody

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A 68-year-old Japanese man developed icteric acute hepatitis during periodic care after undergoing gastrectomy due to early gastric cancer. The routine serological markers for hepatitis A, B and C viruses were all negative. Although the liver enzymes spontaneously recovered without any specific therapy, cholestasis was relatively prolonged and successfully treated with prednisolone. Determination of serum hepatitis E virus (HEV) RNA revealed the transient infection of HEV, and both immunoglobulin (Ig)A and IgG class anti-HEV antibodies were detected after the disease onset, whereas those were negative when measured 3 weeks prior to the onset. In addition, the titer of serum IgA class antibody was associated with the clinical signs of hepatitis. In contrast, no IgM class antibody was detected throughout the course. This case suggests that screening only with IgM class antibody is not sufficient to detect acute HEV infection.

Key words: acute hepatitis E, enzyme-linked immunoassay, immunoglobulin A anti-hepatitis E virus antibody, immunoglobulin M anti-hepatitis E virus antibody

INTRODUCTION

HEPATITIS E VIRUS (HEV) infection is a locally acquired cause of acute hepatitis.[1] HEV is considered to account for a small percentage of overt acute hepatitis in Japan.[2] A practical diagnostic system using serological markers has been developing. Immunoglobulin (Ig)M class antibody (Ab) against a pathogen is generally considered to be a marker for a recent primary infection of the pathogen and it is used as a screening test to determine the etiology of infectious disease.

Twenty cases of acute hepatitis E have been diagnosed in this institute since 1995, primarily by detecting hepatitis E RNA in the serum, and supplementarily by IgA, IgM and IgG class anti-HEV Ab.[3] This report represents one case with overt acute hepatitis E that showed negative result for the IgM anti-HEV from the preclinical period to the remission period, despite the transient detection of HEV RNA in the serum. This finding suggests that screening only with IgM class Ab for HEV may therefore occasionally overlook acute hepatitis E.

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CASE REPORT

A 68-YEAR-OLD Japanese man was referred and admitted to the gastroenterology and hepatology department from the surgery department of Iwate Medical University Hospital because of general fatigue and anorexia with liver injury. He had undergone subtotal gastrectomy 4 months prior to admission because of early gastric cancer. He presented hypertension and arrhythmia at the time of surgery. He remained under the care of the outpatient hospital of the surgery department with a periodic (once a month) checkup and a prescription for herbal relaxants (*da jian zhong tang* and *sennoside*), magnesium carbonate, valsartan and cibenzoline for 4 months before admission. He showed no abnormal findings in physical examinations and