Here we report the results of two studies. First, we studied the presence of HEV in plasma samples collected from blood donors showing extremely high ALT levels in Hokkaido, Japan. Subsequently, we expanded the area of investigation to nationwide and studied HEV prevalence in Japanese blood donor samples with elevated ALT levels obtained from all Japan.

MATERIALS AND METHODS

Blood donor samples with elevated ALT levels in Hokkaido

For the preliminary study, we studied the blood donors with elevated ALT levels of 500 IU per L and greater in Hokkaido. There were 1,049,566 blood donations in Hokkaido from April 2000 through March 2003. Of these, 23,827 (2.3%) were disqualified because of an elevated ALT level of 61 IU per L or greater, which was cutoff value in the Japanese Red Cross (JRC). Of these, 41 had an ALT level of 500 IU per L or greater (Table 1). The samples from these 41 donors enrolled in this study were stored below –20°C until testing. The tests for qualitative HEV RNA and/or for antibodies were performed as described below.

Blood donor samples with elevated ALT levels in nationwide Japan

All donor samples (n = 1389) with ALT levels higher than 200 (mean ± standard deviation [SD], 314 ± 249) IU per L were collected from all JRC Blood Centers over Japan between April 2003 and March 2004. In addition, 1062 donor samples with ALT levels of 61 to 199 IU per L were collected randomly from 3 blood centers (Hokkaido, Hiroshima and Fukuoka). The 47 blood centers were divided into eastern Japan (three blocks: Hokkaido, Miyagi, and Tokyo) and western Japan (four blocks: Aichi, Osaka, Okayama, and Fukuoka; Fig. 1). Hiroshima and Fukuoka blood centers belong to western Japan. The samples were subjected to real-time reverse transcription–polymerase chain reaction (RT-PCR) testing for the presence of HEV RNA and enzyme-linked immunosorbent assay (ELISA) for antibody tests against HEV as described below. The samples were kept frozen below –20°C until testing.

Real-time RT-PCR for HEV RNA detection and sequence analyses

Total nucleic acids were extracted from 200 µL of plasma sample using a virus spin kit (QIAamp MinElute, Qiagen K.K., Tokyo, Japan) according to the manufacturer's instructions. The 20-µL eluate was subjected to one-step real-time RT-PCR and quantitative assay for HEV RNA as described in our previous study.[12] The amplification products were then sequenced directly on both strands and were analyzed as described previously.[16] The amplification products of ORF2 (412 nucleotides) from HEV RNA–positive samples were sequenced and compared with those of reported swine HEV isolates from pigs or pig livers by using GenBank Basic Local Alignment Search Tool (BLAST) homology search at the National Center for Biotechnology Information server (http://www.ncbi.nlm.nih.gov).

The nucleotide sequence data reported in this article will appear in DDBJ/EMBL/GenBank nucleotide sequence databases with the Accession Numbers AB434132 for HRC-HE1, AB434133 for HRC-HE2, AB434134 for HRC-HE3, AB434135 for HRC-HE4, AB434136 for HRC-HE5, AB434137 for HRC-HE6, AB434138 for HRC-HE7, AB434139 for HRC-HE8, AB434140 for HRC-HE9, AB434141 for HRC-HE10, AB434142 for HRC-HE11, AB434143 for HRC-HE12, AB434144 for JRC-HE1, AB434145 for JRC-HE2, AB434146 for JRC-HE3, AB434147 for JRC-HE4, AB434148 for JRC-HE5, AB434149 for JRC-HE6, AB434150 for JRC-HE7, AB434151 for JRC-HE8, AB434152 for JRC-HE9, AB434153 for JRC-HE10, and AB434154 for JRC-HE11.

TABLE 1. ALT-disqualified donors from April 2000 through March 2003 in Hokkaido, Japan (total number of donors, 1,049,566)

	Number of donors with each ALT level (IU/L)						
Donors	61-99	100-199	200-299	300-399	400-499	500-	Total
Male	16,809	3,714	226	35	11	29	20,824
Percent*	88.1	85.8	78.7	60.3	52.4	70.7	87.4
Percent†	1.60	0.35	0.02	0.00	0.00	0.00	1.98
Female	2,281	616	61	23	10	12	3,003
Percent*	11.9	14.2	21.3	39.7	47.6	29.3	12.6
Percent†	0.22	0.06	0.01	0.00	0.00	0.00	0.29
Total	19,090	4,330	287	58	21	41	23,827
Percent†	1.82	0.41	0.03	0.01	0.00	0.00	2.27
Percent‡	80.1	18.2	1.2	0.2	0.1	0.2	100.0

* Rate relative to the donors with each ALT level, showing the ratio of sex difference.
† Rate relative to the total donors (1,049,566).
‡ Rate relative to the ALT-disqualified donors (23,827).

ELISA for HEV antibodies

Purified HEV Genotype 1 virus-like particles derived from recombinant baculovirus-infected insect cells were used as antigens for detection of antibodies to HEV.[17,18] HEV RNA–positive samples from 41 donors enrolled in the preliminary study were assayed by commercial HEV antibody ELISA kit (Cosmic Corp., Ltd., Tokyo, Japan) which basically consisted of the recombinant ORF2 protein as the antigen according to the manufacturer's protocol. In the subsequent study of all samples (n = 1389 and 1062) from all areas of

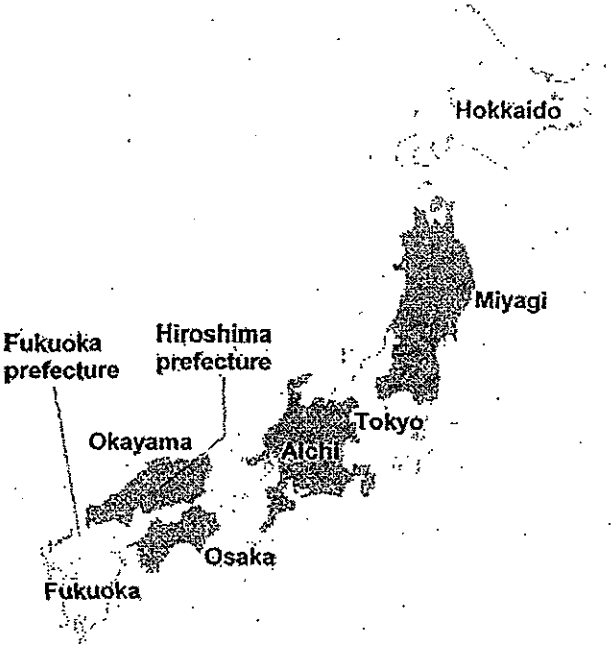

Fig. 1. Map of Japan showing the locations of seven geographic blocks. The 47 blood centers were divided into eastern Japan (three blocks: Hokkaido, Miyagi [six prefectures], and Tokyo [nine prefectures]) and western Japan (four blocks: Aichi [eight prefectures], Osaka [six prefectures], Okayama [nine prefectures] including Hiroshima prefecture, and Fukuoka [eight prefectures] including Fukuoka prefecture).

Japan, ELISA was performed as follows. Wells of microplates (Number 2592, 96-well Stripwell, flat bottom, Corning Life Sciences, Corning, NY) were coated with 50 µL of the recombinant ORF2 protein (3 µg/mL in phosphate-buffered saline [PBS]), and the plates were incubated at room temperature (RT) for 2 hours followed by incubation with 100 µL of blocking buffer containing 40 percent (vol/vol) calf serum (Gibco-BRL, Tokyo, Japan) at RT for 1 hour. The blocking buffer was discarded, and each well was washed five times with 450 µL of washing buffer (0.05% Tween 20 in PBS). To test for anti-HEV immunoglobulin G (IgG), 50 µL of each sample was added to each well at a dilution of 1:100 in saline containing 40 percent calf serum. The microplates were incubated at RT for 1 hour and then washed five times with washing buffer. Fifty microliters of horseradish peroxidase–conjugated goat anti-human IgG (IGB22; Institute of Immunology Co., Ltd., Tokyo, Japan; 1:2000) or immunoglobulin M (IgM; IGM49, Institute of Immunology Co., Ltd.; 1:500) in PBS containing 25 percent (vol/vol) fetal calf serum (PAA Laboratories GmbH, Pasching, Austria) was added to each well and incubated at RT for 1 hour. The wells were washed five times with washing buffer. Fifty microliters of tetramethylbenzidine soluble reagent (Dako Co., Ltd., Carpinteria, CA) as a substrate was added to each well. The plate was incubated at RT for 10 minutes in the dark, and then 50 µL of 1 N sulfuric acid (Kanto Chemical Co., Inc., Tokyo, Japan) as tetramethylbenzidine stop buffer was added to each well. The optical density (OD) of each sample was read at 450 nm. Test samples with OD values equal to or greater than the cutoff value were considered positive for the presence of anti-HEV IgG or anti-HEV IgM in this ELISA. ODs of 0.18 [mean (0.019) + 7 × SD (0.024)] for anti-HEV IgG, and that of 0.19 [mean (0.022) + 6 × SD (0.028)] for anti-HEV IgM were used as the cutoff values. Reactive samples were tested by another HEV antibody ELISA kit (Cosmic) described previously. Samples were determined as positive if they were reactive by both ELISA methods.

Statistical analysis

A two-sided Fisher's exact test was used to compare the percentages of subjects with each HEV marker in the two geographic groups (eastern Japan vs. western Japan) or two age groups (10s-30s vs. 40s-60s).

RESULTS

Prevalence of HEV RNA in donors with elevated ALT levels in Hokkaido

In the primary study, more than 98 percent of those disqualified donors had an ALT level of less than 200 IU per L and more than 87 percent were male (Table 1). The number of donors with elevated ALT levels higher than 500 IU per L was 41 (0.2%). Among the 41 donors, HEV RNAs were detected in 8 (19.5%). Of these, 6 samples were described in our previous study.[9]

Prevalence of HEV RNA in donors with elevated ALT in Japan

Thereafter, we studied a nationwide survey for HEV prevalence in Japanese blood donor samples with elevated ALT levels including levels of less than 500 IU per L, obtained from all Japan. Of 5,621,096 blood donations in 47 blood centers from April 2003 through March 2004, a total of 114,583 (2.0%) were disqualified because of elevated ALT levels of higher than 61 IU per L. Of these, 1389 donors (men vs. women, 5.5 vs. 1; age, 32 ± 11 years [mean ± SD]) showed elevated ALT level of higher than 200 IU per L. A total of 1062 donors with an ALT level of 61 to 199 IU per L were randomly collected from three blood centers as described.

The results are summarized in Table 2 and Fig. 2. Of 1389 donor samples with elevated ALT levels higher than 200 IU per L, 15 (1.1%) were HEV RNA–positive. Although the HEV-positive donor samples were found in any block of Japan, they tended to be more frequent in eastern Japan

TABLE 2. Prevalence of HEV RNA, IgM anti-HEV, and IgG anti-HEV among elevated ALT donors from April 2003 through March 2004 in Japan (total number of donors, 5,621,096)

	ALT levels (61-199 IU/L)					ALT levels (200- IU/L)				
Geographic blocks	Number of donors*	Number RNA-positive (%)	Number IgM-positive (%)	Number IgG-positive (%)	Number of donors†	Number RNA-positive (%)	Number IgM-positive (%)	Number IgG-positive (%)
Hokkaido	364	0 (0.0)	1 (0.3)	21 (5.8)	87	4 (4.6)	3 (3.4)	6 (6.9)
Miyagi	NA	NA	NA	NA	143	3 (2.1)	3 (2.1)	3 (2.1)
Tokyo	NA	NA	NA	NA	335	4 (1.2)	3 (0.9)	19 (5.7)
Aichi	NA	NA	NA	NA	223	1 (0.4)	2 (0.9)	6 (2.7)
Osaka	NA	NA	NA	NA	234	1 (0.4)	1 (0.4)	3 (1.3)
Okayama	345	0 (0.0)	0 (0.0)	7 (2.0)	188	1 (0.5)	1 (0.5)	1 (0.5)
Fukuoka	353	0 (0.0)	0 (0.0)	1 (0.3)	179	1 (0.6)	1 (0.6)	7 (3.9)
Total	1062	0 (0.0)	1 (0.1)	29 (2.7)	1389	15 (1.1)	14 (1.0)	45 (3.2)
(95% CI)			(0.0-0.5)	(1.8-3.9)		(0.6-1.8)	(0.6-1.7)	(2.4-4.3)

* Random sampling of donors with elevated ALT (61-199 IU/L) from three prefectures (Hokkaido, Hiroshima, and Fukuoka).
† All donor samples with elevated ALT levels of higher than 200 IU per L during this period.
CI = confidence interval; NA = not available.

(Hokkaido, Miyagi, and Tokyo; p = 0.015). No HEV RNA–positive sample was detected in 1062 donors with elevated ALT levels of 61 to 199 IU per L. The results indicate that HEV RNA–positive donors with elevated ALT levels higher than 200 IU per L were widely distributed over Japan and the prevalence was the highest in Hokkaido.

Antibodies against HEV in donors with elevated ALT levels in Japan

Of 1389 donor samples with elevated ALT levels higher than 200 IU per L, 14 samples (1.0%) were positive for the presence of IgM antibodies to HEV. Donors with IgM anti-HEV were also frequently found in eastern Japan (p = 0.099) and associated with positive HEV RNA (Table 2). Of 1062 donor samples with elevated ALT levels of 61 to 199 IU per L, only 1 sample was positive for the presence of IgM anti-HEV.

Of 1389 donor samples with elevated ALT levels higher than 200 IU per L, 45 samples (3.2%) were positive for the presence of IgG anti-HEV. Again, donors with IgG anti-HEV were more frequent in eastern Japan (p = 0.003) and not associated with HEV RNA–positive donors (Table 2). The frequency of IgG anti-HEV–positive donors appeared to be age-dependent, that is, from 0 percent of donors in their 10s to 12.5 percent of donors in their 60s (10s-30s vs. 40s-60s; p < 0.0001; Fig. 2). Of 1062 donor samples with elevated ALT levels of 61 to 199 IU per L, 29 samples (2.7%) were positive for the presence of IgG anti-HEV (Table 2). Again, the IgG anti-HEV–positive donors were more frequent in eastern Japan (p < 0.0001) and it appeared to be age-dependent (10s-30s vs. 40s-60s; p = 0.001, data not shown).

Analysis for HEV RNA–positive donors

We verified in detail the HEV RNA–positive samples obtained from two studies. Results of analyses for 8 (ALT ≥ 500 IU/L from Hokkaido) and 15 (ALT ≥ 200 IU/L from Japan) HEV RNA–positive donors are summarized in Table 3. The ensuing investigation revealed that all had no history of recent travel in HEV-endemic areas and remained asymptomatic despite of their elevated ALT levels. The concentration of HEV RNA varied from 1.9 to 7.5 log copies per mL. Of the 23 samples, 3 were seronegative, 2 were IgM anti-HEV–positive, 17 were IgM/IgG anti-HEV–positive, and 1 were IgG anti-HEV–positive samples. Twenty-three HEV RNA–positive samples were segregated into Genotype 3 (n = 19) and Genotype 4 (n = 4). These constituted 21 males and 2 females ages 25 to 62 years. Some of the 23 HEV RNA–positive donors were repeat donors. The results of the tests with samples from their other donations revealed that HEV RNA was detected in the previous donation in Donor 12 (HRC-HE12). The sample was negative for the presence of both IgM and IgG

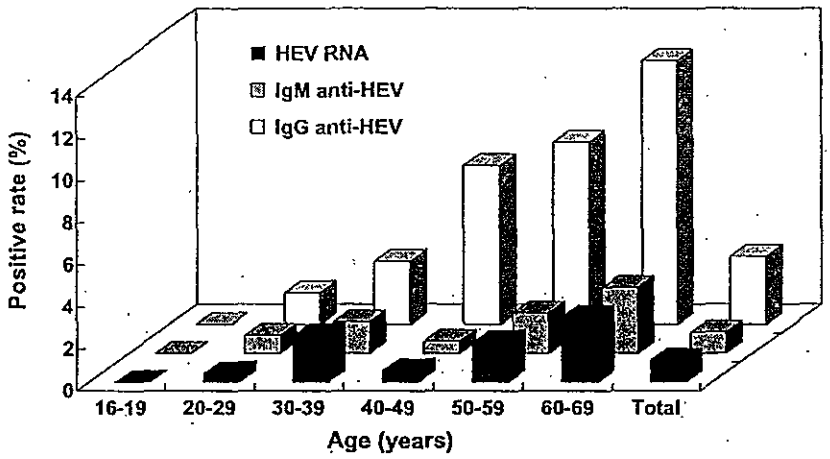

Fig. 2. Age-specific prevalence rates of HEV RNA (■), IgM anti-HEV (▨), and IgG anti-HEV (□) in Japanese donors with elevated ALT levels of 200 IU per L and greater from April 2003 through March 2004. The total number of tested donors was 1389.

anti-HEV with normal ALT. The donated blood (whole blood) was not used for transfusion, because of the low volume of red cells. The plasma was in quarantine. Except for Donor 12, neither HEV RNA nor anti-HEV was detected in other donations.

When the 412-nucleotide ORF2 partial sequences of the HEV-positive 23 isolates were compared with those of reported HEV isolates from pigs or pig livers of Japan, all had a high nucleotide sequence identity of higher than 92.2 percent. More specifically, HRC-HE8 and JRC-HE5 had the highest nucleotide sequence identity, of 99.8 percent, with swJ11-4 and swJ19-1, respectively. Also, JRC-HE1, HRC-HE12, and HRC-HE3 had 99.3, 99.3, and 98.8 percent identities with swJ18-3, swJ13-1, and swJL145, respectively (Table 3).

DISCUSSION

The aim of this study was to investigate the prevalence of HEV among elevated ALT blood donors in Japan. The results of the primary study suggest that HEV was a major causative agent among blood donors with ALT levels higher than 500 IU per L in Hokkaido, since we demonstrated that HEV RNA was detected in 8 of 41 (19.5%) of the high ALT donor samples. Subsequently, a nationwide survey for HEV prevalence in blood donor samples with elevated ALT from all JRC revealed that 1.1 percent (n = 15) of donor samples with elevated ALT levels higher than 200 IU per L were positive for the presence of HEV RNA. No HEV RNA–positive samples were detected in donor samples with elevated ALT levels of 61 to 199 IU per L. Although the 15 HEV RNA–positive donors were widely distributed over Japan, they were frequently found in eastern Japan, especially in Hokkaido (4/15), Miyagi (3/15), and Tokyo (4/15).

It should be noted that in Hokkaido, 8 of the 41 donors with ALT levels of 500 IU per L or greater were positive for the presence of HEV, which is known to be transmitted by transfusion. Thus, as a result of performing HEV tests as the following study among 124 blood donors with ALT levels of 200 to 499 IU per L in Hokkaido, 1 donor (0.8%) was HEV RNA–positive (data not shown). Based on these results, in the subsequent study we expanded the area of investigation to nationwide and studied HEV prevalence in Japanese blood donor samples with elevated ALT including levels of less than 500 IU per L, obtained from all Japan. As for the geographical distribution of hepatitis E in Japan, it was reported that there was a higher prevalence of HEV-infected donors in the eastern part of Japan (Hokkaido, Miyagi, and Tokyo blocks).[15] We cannot clearly explain the reason why blood donors with HEV markers were more frequent in eastern than western Japan. Further studies with a larger number of donors including normal ALT levels will be necessary to draw a definitive conclusion.

Twenty-three HEV RNA–positive samples were divided into Genotype 3 (n = 19) and Genotype 4 (n = 4). Because it is commonly assumed that blood donors are healthy adults, most of those HEV-positive donors appeared to be asymptomatic. Since the isolates of acute hepatitis E patient samples were predominantly Genotype 4 in Japan,[19] the genotypes may play an important role in clinical progression of HEV infection. HEV-positive donors with ALT levels higher than 500 IU per L appeared to be asymptomatic and their ALT elevation was transient (unpublished observation).

In this study, the routes of HEV transmission of infected donors are not clear. The HEV RNA–positive donors had no history of recent travel abroad in areas where HEV is hyperendemic. Yazaki and his colleagues[4] reported that of the 363 packages of raw pig liver sold in grocery stores as food in Hokkaido, 7 (1.9%) packages had detectable HEV RNA. In this study, some isolates from the HEV RNA–positive donor samples showed close sequence homology with the isolates from pigs in Japan, suggesting that HEV transmission may be associated with the consumption of undercooked or inadequately cooked pig meat. Emerson and colleagues[20] reported that some HEV would most likely survive the internal temperatures of rare-cooked meat. When the 412-nucleotide ORF2 partial sequences of the 23 HEV RNA–positive donor isolates were compared with those of reported HEV isolates from pigs or pig livers of Japan, at least 9 isolates (39%) showed close sequence homology (98.5%-99.8%) with the

TABLE 3. Profile of HEV RNA–positive donors

Donor*	Geographic blocks	Date of donation	Age (years)	Sex	ALT (IU/L)	HEV RNA (log copies/mL)	Anti-HEV IgM	Anti-HEV IgG	HEV genotype	Strain	HEV strain with the highest homology among the known swine isolates [Accession No.] (%)†		
1	Hokkaido	Dec. 2000	29	M	767	5.6	+	+	4	HRC-HE1	swJL145‡	[AB105902]	(98.5)§
2	Hokkaido	Mar. 2001	30	M	506	5.0	+	+	3	HRC-HE2	swJHR1-1	[AB194528]	(93.9)
3	Hokkaido	Apr. 2001	40	M	1,470	6.9	+	+	4	HRC-HE3	swJL145‡	[AB105902]	(98.8)§
4	Hokkaido	Jul. 2001	47	M	713	5.1	+	+	3	HRC-HE4	swJTT1-1	[AB194526]	(93.4)
5	Hokkaido	Oct. 2001	62	M	2,080	6.3	+	+	3	HRC-HE9	swJL234‡	[AB105903]	(98.5)§
6	Hokkaido	Oct. 2001	39	M	641	5.1	+	+	3	HRC-HE5	swJL234‡	[AB105903]	(98.5)§
7	Hokkaido	Nov. 2001	48	M	740	3.6	+	+	4	HRC-HE6	swJL145‡	[AB105902]	(98.5)§
8	Hokkaido	Feb. 2003	39	F	578	6.2	–	+	3	HRC-HE7	swJL234‡	[AB105903]	(96.1)
9	Hokkaido	Jul. 2003	35	M	575	5.0	+	+	3	HRC-HE8	swJ11-4‡	[AB094243]	(99.8)§
10	Hokkaido	Oct. 2003	38	M	244	3.4	–	–	3	HRC-HE10	swJHK5-1‡	[AB194486]	(95.4)
11	Hokkaido	Nov. 2003	52	M	576	3.9	+	+	3	HRC-HE11	swJL234‡	[AB105903]	(96.1)
12	Hokkaido	Jan. 2004	38	M	793	5.9	+	+	4	HRC-HE12	swJ13-1‡	[AB094254]	(99.3)§
13	Miyagi	Dec. 2003	39	M	470	5.4	+	+	3	JRC-HE4	swJ24-1	[AB094306]	(92.5)
14	Miyagi	May 2003	25	M	222	4.2	+	+	3	JRC-HE6	swJL234‡	[AB105903]	(95.1)
15	Miyagi	Jan. 2004	34	M	273	3.8	+	+	3	JRC-HE7	swJ2-1‡	[AB094207]	(92.7)
16	Tokyo	Mar. 2004	41	F	216	1.9	+	+	3	JRC-HE9	swJAK6-2	[AB194512]	(93.7)
17	Tokyo	Jun. 2003	34	M	211	3.1	+	+	3	JRC-HE5	swJ19-1	[AB094279]	(99.8)§
18	Tokyo	Nov. 2003	34	M	447	6.8	–	–	3	JRC-HE1	swJ18-3	[AB094277]	(99.3)§
19	Tokyo	Feb. 2004	36	M	328	5.2	+	–	3	JRC-HE10	swJC1990	[AB096756]	(92.7)
20	Aichi	Feb. 2004	62	M	281	3.9	+	+	3	JRC-HE11	swJSZ1-1	[AB194524]	(92.2)
21	Osaka	Mar. 2004	37	M	793	5.9	–	–	3	JRC-HE8	swJHR1-1	[AB194528]	(95.9)
22	Okayama	May 2003	29	M	554	5.3	+	+	3	JRC-HE2	swJIW4-1	[AB194496]	(92.7)
23	Fukuoka	Aug. 2003	57	M	398	7.5	+	–	3	JRC-HE3	swJHR1-1	[AB194528]	(93.4)

* HEV RNA–positive donors: samples from Donors 1 through 8 were obtained from the primary study (ALT ≥ 500 IU/L from Hokkaido) and Donors 9 through 23 from the secondary study (ALT ≥ 200 IU/L from all Japan).
† Nucleotide sequences were compared to the GenBank databases utilizing the BLAST program available at http://www.ncbi.nlm.nih.gov as of March 2008.
‡ Isolates from Hokkaido.
§ Identities of 412-nucleotide ORF2 sequences over 98.5 percent are indicated.
+ = positive; – = negative; M = male; F = female.