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Isolation of epizootic hemorrhagic disease virus serotype 7 from cattle showing fever in Japan in 2016 and improvement of a reverse transcription-polymerase chain reaction assay to detect epizootic hemorrhagic disease virus

Kunitaka YAMAMOTO[1)], Riki HIROMATSU[1)], Mina KAIDA[1)], Tomoko KATO[2)], Tohru YANASE[2)] and Hiroaki SHIRAFUJI[2)]*

[1)]Fukuoka Chuo Livestock Hygiene Service Center, 4-14-5 Hakozaki-Futo, Higashi-ku, Fukuoka 812-0051, Japan
[2)]Kyushu Research Station, National Institute of Animal Health, National Food and Agriculture Research Organization (NARO), 2702 Chuzan, Kagoshima 891-0105, Japan

ABSTRACT. Epizootic hemorrhagic disease (EHD) is an arthropod-borne disease of wild and domestic ruminants caused by the EHD virus (EHDV). To date, seven EHDV serotypes have been identified. In Japan, strain Ibaraki of EHDV serotype 2 has caused outbreaks of Ibaraki disease in cattle. In addition, EHDV serotype 7 (EHDV-7) has caused large-scale EHD epizootics. In mid-September 2016, eight cattle at a breeding farm in Fukuoka Prefecture, Japan developed fever. Since EHDV-7 was detected in sentinel cattle in western Japan in 2016, we suspected that the cause of this fever might be an EHDV-7 infection. In this study, we tested cattle for EHDV-7 and some other viruses. Consequently, EHDV was isolated from washed blood cells collected from three of the eight cattle, and genetic analysis of genome segment 2 revealed that this isolate was EHDV-7. Moreover, all affected cattle tested positive for anti-EHDV-7 neutralizing antibodies. Our results suggest that the fever was caused by EHDV-7 infection. In addition, we modified a conventional reverse transcription polymerase chain reaction assay for the specific detection of EHDV. This modified assay could detect various strains of EHDV isolated in Japan, Australia, and North America. Furthermore, the assay permitted the detection of EHDV-7 in blood cells collected from seven of the eight cattle. We believe that this modified assay will be a useful tool for the diagnosis of EHD.

KEY WORDS: arbovirus, cattle, epizootic hemorrhagic disease, febrile illness, *Orbivirus*

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Epizootic hemorrhagic disease (EHD) is an arthropod-borne disease of wild and domestic ruminants caused by the EHD virus (EHDV; genus *Orbivirus*, family *Reoviridae*) [21, 31]. EHDV is transmitted by *Culicoides* biting midges, and EHDV infection primarily affects white-tailed deer (*Odocoileus virginianus*) [21, 31]. EHDV infection in cattle usually does not result in clinical disease, but some EHDV-infected cattle show fever, anorexia, excessive salivation, nasal and ocular discharge, congestion of conjunctival and nasal mucous membranes, swollen eyelids and tongue, and dysphagia [21, 26, 31].

EHDV has a 10-segmented double-stranded RNA (dsRNA) genome encoding seven structural proteins (VP1–VP7) and four non-structural proteins (NS1, NS2, NS3/NS3a, and NS4) [2–5]. Two of the 10 genome segments, Seg-2 and Seg-6, encode the VP2 and VP5 outer-capsid proteins, respectively [3]. The structural protein VP2 is highly variable and correlates with the serotype [3]; VP5 is also variable and shows some correlation with the serotype [3]. Another segment, Seg-3, encodes an inner core protein, VP3 [2]. Phylogenetic analyses of Seg-3 have been conducted to identify geographic genetic types (topotypes) of the bluetongue virus (BTV), which is related to EHDV [27, 28]. Another study demonstrated that phylogenetic analyses of Seg-3 can be used to classify EHDV strains in several groups based on their geographic origins [39].

To date, seven EHDV serotypes have been recognized [21] and two putative serotypes have been reported [19, 33]. In Japan, strain Ibaraki of EHDV serotype 2 (EHDV-2) was first identified in affected cattle in 1959; the strain subsequently caused outbreaks of Ibaraki disease in cattle [17, 26]. Two other EHDV serotypes, EHDV-7 and EHDV-6, have also caused large-scale EHD epizootics. The clinical features of epizootic diseases caused by EHDV-7 in 1997 were mainly abortion and stillbirth in

*Correspondence to: Shirafuji, H.: shirah@affrc.go.jp

addition to febrile illness [23]. In contrast, the main clinical features caused by EHDV-6 in 2015 were very similar to those of typical Ibaraki disease, i.e., fever, anorexia, salivation, and dysphagia [13]. Another serotype, EHDV-1, and a putative serotype, EHDV-10, have been identified in Japan but were isolated from asymptomatic cattle [33]. Several EHDV serotypes have been identified in clinical cases in cattle in a number of other countries, including EHDV-2 in the USA [12]; EHDV-6 in France (Réunion Island), Turkey, and Israel [6, 10, 29, 35]; and EHDV-7 in Israel [16, 40]. EHDV-1 strains have been suspected to cause clinical cases in Israel and Egypt [1, 9].

In the summer of 2016, seroconversion to EHDV-2-positive status occurred in sentinel cattle in the western part of Japan, including Fukuoka Prefecture. Although infection with EHDV-2 was suspected, only EHDV-7 was detected in the seroconverted animals. Given the antigenic cross-reactivity between EHDV-2 and EHDV-7 [3], the seroconversion to EHDV-2-positive status in 2016 is inferred to have likely indicated the circulation of EHDV-7, and not EHDV-2, in cattle. In mid-September 2016, six cattle at a breeding farm in Fukuoka Prefecture were reported to have developed fever. While EHDV-2 infection has been the main cause of EHD in Japan, we suspected that the cause of this fever might be EHDV-7 infection. Therefore, we examined the cattle to clarify whether the infection was caused by EHDV-7, considering that EHDV is an important arbovirus that can cause economic losses in the livestock industry. Here, we report the results of a 24-day survey performed on nine cattle, eight of whom exhibited fever, including the genetic characterization of a virus strain isolated from these cattle. Additionally, we describe the modification of a reverse transcription-polymerase chain reaction (RT-PCR) assay for the specific detection of EHDV in bovine blood.

MATERIALS AND METHODS

Rectal temperature measurement and blood sample collection

Rectal temperatures of nine cattle (2–8 years old) were measured four times: on September 17 (day 0), September 25 (day 8), October 3 (day 16), and October 11 (day 24). Blood samples were collected from the nine cattle on September 20 (day 3) and October 6 (day 19). On day 3, heparinized blood samples were also obtained for virus isolation, and these samples were centrifuged at 1,940 × *g* for 15 min at 4°C. The resulting supernatants (plasma) were stored at −80°C until analysis; the pelleted blood cells were washed three times with sterilized phosphate-buffered saline (PBS), resuspended in PBS, and stored at −80°C until analysis. Whole blood samples were collected without anticoagulant on days 3 and 19 for virus neutralization tests. These samples were allowed to clot and subjected to centrifugation; the resultant serum samples were stored at −20°C until analysis.

Virus isolation

The plasma and washed blood cells collected from five of the nine cattle (Nos. 1–5) were used for virus isolation. The samples were inoculated onto baby hamster kidney (BHK-21) and hamster lung (HmLu-1) cells as previously described [14]. Briefly, cells were incubated in test tubes with Eagle's minimum essential medium (MEM; Nissui, Tokyo, Japan) supplemented with 0.295% tryptose phosphate broth (Becton Dickinson and Co., Franklin Lakes, NJ, USA), 0.015% sodium bicarbonate, and 10% bovine serum overnight at 37°C. The cells were washed three times with Earl's solution before inoculation. The cell cultures inoculated with blood samples were maintained in serum-free medium by rotation at 37°C for 7 days and were collected if a cytopathic effect (CPE) was observed. If a CPE was not observed, two further blind passages were conducted in the same manner. The supernatant of the cell culture showing a CPE was collected and was further subjected to RNA extraction using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The RNA samples were used as templates for a multiplex RT-PCR assay targeting several arboviruses, including EHDV [24].

Full-length amplification of cDNAs (FLAC) and Sangar and high-throughput sequencing

Full-length cDNA of each EHDV segment for sequence analysis was prepared as described previously [20, 32, 33] with some modifications. Briefly, dsRNA was isolated from EHDV-infected HmLu-1 cells and showed approximately 90% CPE. Each of the cDNA segments was synthesized from purified EHDV dsRNA using the sequence-independent method FLAC.

For Seg-2, -3, and -6, the viral dsRNA segments were used as templates for cDNA synthesis using ThermoScript RT (Invitrogen/Thermo Fisher Scientific, Carlsbad, CA, USA). The synthesized cDNAs were amplified by PCR using primers 5-15-1 (GAGGGATCCAGTTTAGAATCCTCAGAGGTC) and KOD -Plus- Ver. 2 DNA polymerase (Toyobo, Otsu, Japan); the products were subjected to direct sequencing.

For the remaining seven segments (Seg-1, Seg-4, Seg-5, Seg-7, Seg-8, Seg-9, and Seg-10), viral dsRNA segments were used for next-generation sequencing (NGS). The dsRNA was subjected to double-stranded cDNA synthesis using the NEBNext RNA Ultra First Strand Synthesis Module and the NEBNext RNA Ultra Second Strand Synthesis Module (New England BioLabs, Ipswich, MA, USA). The resulting cDNA was used to prepare libraries for NGS processing using a TruSeq DNA Nano LT Library Prep Kit (Illumina, San Diego, CA, USA). The libraries were then loaded on an iSeq 100 i1 Reagent (Illumina) and sequenced on an iSeq 100 (Illumina). The sequence reads were subjected to *de novo* assembly using CLC Genomics Workbench 12 (Qiagen), and possible viral sequences were identified from contigs by comparison with sequences in GenBank using BLASTx. Probable gaps between the obtained contigs were amplified by RT-PCR, and the products were sequenced using BigDye Terminator v 3.1 (Thermo Fisher Scientific, Waltham, MA, USA) on an ABI 3100-Avanti Genetic Analyzer (Thermo Fisher Scientific). The nucleotide sequence data reported herein have been deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers LC552731−LC552733 and LC599904–LC599917.

Phylogenic analysis

To analyze phylogenetic relationships among the sequenced virus strains and database sequences, the sequences were aligned using ClustalW [36]. Phylogenetic trees were constructed using MEGA X software (https://www.megasoftware.net/) with the maximum-likelihood method [18, 34]. The reliability of branching orders was evaluated using a bootstrap test with 1,000 replicates. Sequence identities among the strains were calculated using GENETYX ver. 15 (Genetyx, Tokyo, Japan).

EHDV detection in blood samples using RT-PCR assays

Washed blood cells collected from eight of the nine cattle (Nos. 1–7 and 9) were used for RNA extraction. The RNA was extracted using an RNeasy Mini Kit, and RNA samples were used as templates for two RT-PCR assays: the previously reported multiplex RT-PCR assay [24] and a modified RT-PCR assay with a newly designed primer pair (EHDV/S3/1380-1399F and EHDV/S3/2053-2034R; Table 1). The new primer pair was designed based on Seg-3 of the EHDV strain isolated in the present study (as well as Seg-3 sequences of EHDV strains isolated elsewhere). The target amplicon size of the modified assay (with the new primers) was 674 bp.

Using the new primer pair and a OneStep RT-PCR Kit (Qiagen), the RT-PCR assay was conducted under the following conditions: 50°C for 30 min (reverse transcription); 95°C for 15 min (activation of *Taq* DNA polymerase, inactivation of RT enzyme, and denaturation of the template cDNA); 35 cycles of denaturation/annealing/extension at 94°C for 30 sec, 55°C for 30 sec, and 68°C for 1 min; and 68°C for 7 min (final extension).

Validation of the modified RT-PCR assay

The analytical specificity of the modified RT-PCR assay was validated using viral RNA extracted from each of the 21 EHDV strains (Table 2) and nine other arboviruses (Akabane, Aino, Peaton, Sathuperi, Shamonda, Chuzan, D'Aguilar, bovine ephemeral fever, and BTV). For verification of the analytical sensitivity, *in vitro* transcribed RNA fragments of the EHDV strains FO-1/E/16 (an EHDV strain obtained in this study) and KS-8/E/13 (EHDV-2) were used as described previously [22]. The cDNA fragment was synthesized from genomic RNA of each strain using a primer set, EHDV/S3/1281-1299F and EHDV/S3/2277-2260R (Table 1), and inserted into the pGEM-T Easy vector (Promega, Madison, WI, USA). EHDV RNA fragments were synthesized using the MEGAscript T7 Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Serial 10-fold dilutions of the

Table 1. Oligonucleotide primers used for the development of an RT-PCR assay in this study

Primer	Sequence (5′–3′)	Position in EHDV Seg-3	Product size	Purpose
EHDV/S3/1380-1399F	GTTGAAAATAGAGARCCAGC	1380–1399	674 bp	RT-PCR
EHDV/S3/2053-2034R	ARCTTCATCGAGTTYTGCAT	2053–2034		
EHDV/S3/1281-1299F	CCGCTAGATTTCCGAATAG	1281–1299	997 bp	Cloning
EHDV/S3/2277-2260R	CCATATTCGCTTGTAACC	2277–2260		

Table 2. EHDV strains used for the development of an RT-PCR assay in this study

Strain	Serotype	Year collected	Geographical origin	GenBank accession number (Seg-3)
Kawanabe 525	1	1985	Kagoshima	LC202964
ON-1/B/01	1	2001	Okinawa	LC202968
ON-6/E/10	1	2010	Okinawa	LC202971
ON-1/E/13	1	2013	Okinawa	LC202950
NS-1/P/00	2	2000	Nagasaki	LC202967
SG-1/E/02	2	2002	Saga	LC202969
KS-7/E/13	2	2013	Kagoshima	LC202951
KS-8/E/13	2	2013	Kagoshima	LC202952
KSB-14/E/97	7	1997	Kagoshima	LC202965
ON-23/E/06	7	1998	Okinawa	LC202970
ON-4/B/98	10	1998	Okinawa	LC202966
HG-1/E/15	6	2015	Hyogo	LC320036
ON-1/E/16	7	2016	Okinawa	LC599918
JPN1959/01 Ibaraki	2	1959	Ibaraki	AM745079
AUS1979/01 CSIRO 439	2	1979	Australia	AM744989
AUS1977/01 CSIRO 157	5	1977	Australia	AM745029
AUS1981/07 CSIRO 753	6	1981	Australia	AM745039
AUS1981/06 CSIRO 775	7	1981	Australia	AM745049
AUS1982/06 CPR3961A	8	1982	Australia	AM745059
USA1955/01 New Jersey	1	1955	USA	AM744979
CAN1962/01 Alberta	2	1962	Canada	AM744999

synthesized RNA (10^0–10^6 copies/reaction) were prepared using RNA extracted from EHDV-free bovine blood cells. RNA was used to determine the detection limit of the RT-PCR assay.

Virus neutralization test

Virus neutralization tests were conducted using serum samples, an EHDV strain isolated in the present study, and HmLu-1 cells, as described previously [15]. In addition, neutralizing antibody titers were examined against bovine ephemeral fever virus, bovine herpesvirus 1, bovine parainfluenza virus 3, and bovine viral diarrhea virus types 1 and 2.

RESULTS AND DISCUSSION

Rectal temperatures

Since the owner of the breeding farm noticed that some cattle developed a fever, we measured the rectal temperatures of nine cattle on the farm. Rectal temperatures ≥39.4°C were observed in six cattle on day 0, seven cattle on day 8, and four cattle on day 16. On day 24, rectal temperatures of the nine cattle ranged between 37.9°C and 38.4°C. Three of the nine cattle (Nos. 3, 5, and 7) had rectal temperatures ≥39.4°C on days 0, 8, and 16 (Table 3). Based on the guidelines for fever in cattle [8, 30], the elevation of rectal temperatures in eight cattle (No. 1–7, 9) indicated that these animals had fever within 17 days between Day 0 and Day 16.

Virus neutralization test

Considering that EHDV-7 infection may have been the cause of the fever, we tested the collected serum samples for anti-EHDV-7 neutralizing antibodies. An 8-fold or greater increase in neutralizing antibody titer was observed in five cattle (Nos. 1–5). While no ≥8-fold increase was observed in sera from the other four cattle (Nos. 6–9), the titers of the sera collected from these 4 cattle on the first sample collection day (September 20; day 3) were 1:8 to 1:32 (Table 3). Our data indicate that all nine cattle were infected with EHDV-7 by October 6 (day 19). No increase of ≥4-fold was observed in the neutralizing antibody titers against the other tested viruses in any of the nine cattle examined in the present study (data not shown).

Viral RNA detection in the blood samples

We tested the washed blood cells collected from the eight cattle (Nos. 1–7 and 9) for arboviruses, including EHDV, considering that EHDV may have been the cause of the fever. Three of the eight cattle (Nos. 1–3) tested positive and the other five cattle (Nos. 4–7 and 9) tested negative for EHDV by the multiplex RT-PCR assay; the latter set of five cattle (Nos. 4–7 and 9) also tested negative by a single RT-PCR assay using the primer set for EHDV detection included in the multiplex RT-PCR assay (data not shown). Aside from EHDV, none of the other targeted arboviruses were detected in any of the samples tested by the multiplex RT-PCR assay (data not shown). However, it was possible that the five cattle (Nos. 4–7 and 9) had viremia on day 3 because all of these cattle tested positive for anti-EHDV-7 neutralizing antibodies. Viremia of EHDV can be prolonged for beyond 50 days despite the presence of neutralizing antibodies owing to an intimate association between the virus and erythrocytes [7, 25]. Therefore, to confirm whether the undetectability of EHDV RNA in these five seroconverted cattle was due to mutations in Seg-3, which should be recognized by the primers, the primer sequences were aligned to the Seg-3 sequence of the EHDV strain FO-1/E/16. There were only a few mismatches of two nucleotides in the sense primer and one nucleotide in the antisense primer. However, we designed a new primer pair targeting EHDV Seg-3 because the sensitivity of the RT-PCR assay might be improved by modifying the primer sequences. Thus, we conducted an RT-PCR assay using the new primer pair (designated EHDV/S3/1380-1399F and EHDV/S3/2053-2034R) to detect EHDV in the washed blood obtained from eight of the nine cattle (Nos. 1–7 and 9). Seven of the eight cattle tested positive for EHDV in the modified assay (Table 3).

Table 3. Summary of ages, rectal temperatures, and laboratory findings of the cattle

No.	Age, years	Rectal temperature				RT-PCR[a)]	Virus isolation	Neutralizing antibody titer[b)]	
		17 Sep (day 0)	25 Sep (day 8)	03 Oct (day 16)	11 Oct (day 24)			20 Sep (day 3)	06 Oct (day 19)
1	8	**40.0**	**40.0**	38.6	38.3	+	+	1:2	1:16
2	7	**39.8**	39.0	39.0	37.9	+	–	1:4	1:64
3	6	**39.7**	**39.5**	**39.8**	38.3	+	+	<1:2	1:16
4	3	**40.9**	**39.8**	38.7	38.2	+	+	<1:2	1:32
5	3	**39.5**	**39.9**	**39.6**	38.4	–	–	<1:2	1:16
6	7	38.9	**39.5**	39.0	38.4	+	NT	1:16	1:16
7	3	**39.4**	**39.5**	**39.5**	38.0	+	NT	1:8	1:16
8	2	NT	38.6	39.0	38.4	NT	NT	1:32	1:32
9	6	NT	**40.3**	**39.4**	38.3	+	NT	1:8	1:16

a) A newly designed primer set (EHDV/S3/1380-1399F and EHDV/S3/2053-2034R) was used. b) A neutralization test was conducted using the EHDV-7 strain. FO-1/E/16 was obtained from washed blood cells of the affected cattle (No. 1). NT: not tested. Rectal temperatures ≥39.4°C are shown in bold. EHDV, epizootic hemorrhagic disease virus.

Virus isolation

We collected heparinized blood samples on day 3 to test for fever-causing viruses. After the inoculation of the plasma and washed blood cells onto cultivated cells followed by blind passages, the HmLu-1 cells inoculated with the blood cells of three cattle (Nos. 1, 3, and 4) showed a CPE characterized by rounding, aggregation, and cell detachment. To further clarify whether the CPE was caused by EHDV or other arboviruses, the presence of arbovirus RNA in the culture supernatant was tested using multiplex RT-PCR [24]. Only EHDV RNA was detected in the supernatant from all three inoculated cells (Table 3), indicating that arboviruses other than EHDV did not propagate in these cells.

Genetic characteristics of an EHDV strain

We performed sequence analysis of genome segments 2, 3, and 6 using one of the obtained EHDV isolates (strain FO-1/E/16) to identify its serotype and to understand the relationships between strain FO-1/E/16 and other EHDV strains. Phylogenetic analysis of Seg-2 revealed that the EHDV strain FO-1/E/16 clustered with EHDV-7 strains isolated in Japan, China, and Australia (Fig. 1). Strain FO-1/E/16 showed the highest identity with a Japanese EHDV-7 isolate, strain KSB-14/E/97 (97.29% nucleotide identity, 98.16% amino acid identity). Since Seg-2 encodes the VP2 outer-capsid protein, which correlates with the serotype [3], our data indicate that strain FO-1/E/16 belongs to EHDV-7. Strain FO-1/E/16 also clustered with Japanese and Chinese EHDV-7 strains in the phylogenetic tree based on Seg-6 (Fig. 2). Among these Japanese and Chinese EHDV-7 strains, a Japanese EHDV-7 isolate, strain KSB-14/E/97, showed the highest identity with strain FO-1/E/16 (97.98% nucleotide identity, 99.43% amino acid identity). In contrast, the phylogenetic analysis of Seg-3 showed that the EHDV-7 strain FO-1/E/16 clustered with Japanese, Chinese, and Australian EHDV strains of multiple serotypes belonging to the E1 subgroup (Fig. 3) [33]. Within the E1 subgroup of the Eastern group, the Japanese EHDV-10 strain ON-4/B/98 showed the highest nucleotide identity (96.24%) with strain FO-1/E/16. The EHDV-7 strain FO-1/E/16 was most closely related to Japanese EHDV strains in the aforementioned phylogenetic analysis of the three genome segments; however, overwintering of EHDV and other arboviruses that infect cattle is seemingly unrealistic in Japan [15]. Therefore, the EHDV-7 strain FO-1/E/16 is thought to be a part of EHDVs circulating in the Asia-Pacific region, similar to other Japanese EHDV strains [33].

We then performed sequence analysis of the remaining seven genome segments (Seg-1, Seg-4, Seg-5, Seg-7, Seg-8, Seg-9, and Seg-10). We analyzed these segments to clarify the difference in genetic characteristics between the EHDV-7 strain FO-1/E/16 and the latest EHDV-2 strain KS-8/E/13 that caused typical Ibaraki disease in 2013 [11]. The dsRNA samples of the EHDV-7 strain FO-1/E/16 and EHDV-2 strain KS-8/E/13 were subjected to NGS. Characteristics of all genome segments are summarized in Table 4. The two strains had the same segment lengths and predicted protein sizes, and the respective genome segments exhibited the same 5′-terminal hexanucleotides, 5′-non-coding region (NCR) lengths, and 3′-NCR lengths. The only difference was that the first

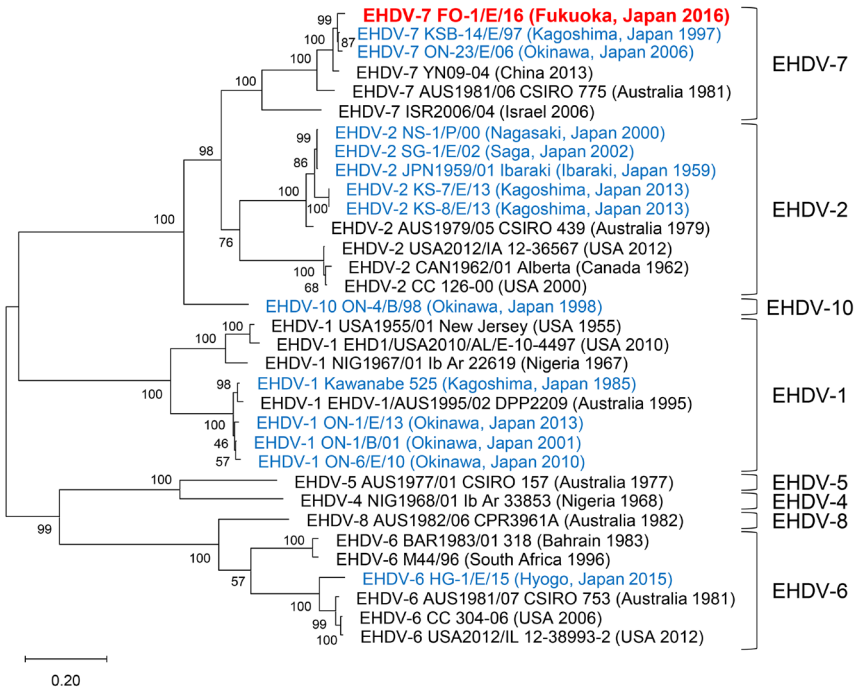

Fig. 1. Phylogenetic profile showing relationships among the epizootic hemorrhagic disease virus (EHDV) strains based on nucleotide sequences of the full-length coding region of genome segment 2. The EHDV-7 strain FO-1/E/16 isolated in this study is shown in red, and other Japanese EHDV strains are shown in blue. The percentage bootstrap values calculated from 1,000 replicates are indicated around the internal nodes. The scale bar represents 0.20% sequence divergence.

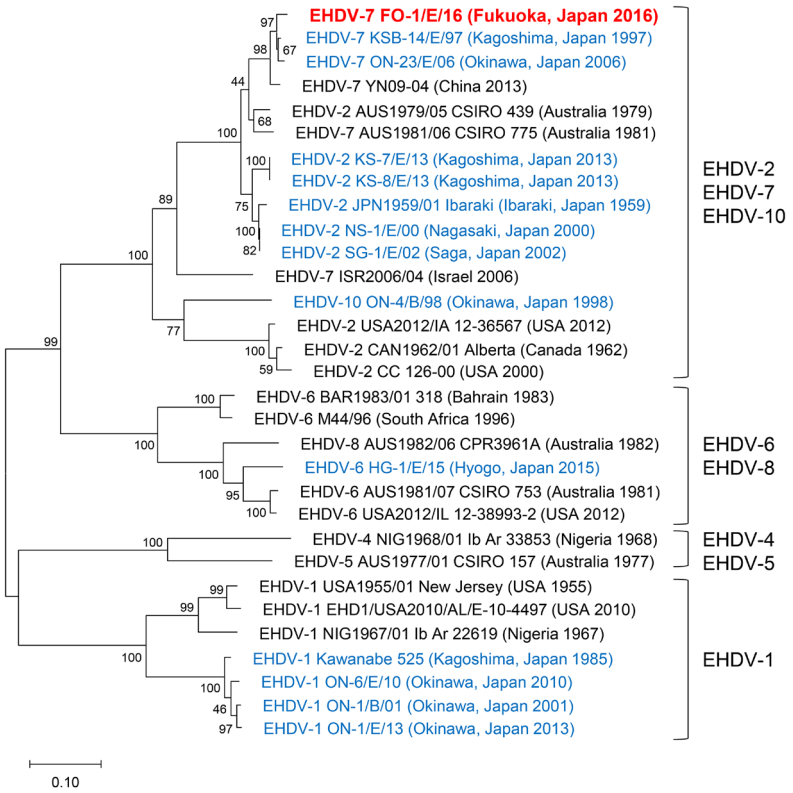

Fig. 2. Phylogenetic profile showing relationships among the epizootic hemorrhagic disease virus (EHDV) strains based on nucleotide sequences of the full-length coding region of genome segment 6. The EHDV-7 strain FO-1/E/16 isolated in this study is shown in red, and other Japanese EHDV strains are shown in blue. The percentage bootstrap values calculated from 1,000 replicates are indicated around the internal nodes. The scale bar represents 0.10% sequence divergence.

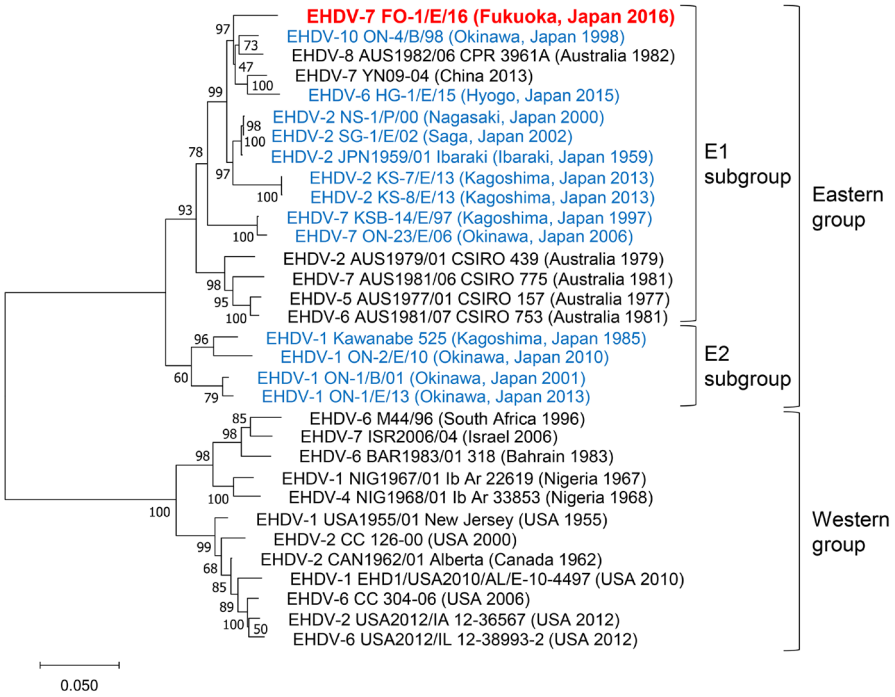

Fig. 3. Phylogenetic profile showing relationships among the epizootic hemorrhagic disease virus (EHDV) strains based on nucleotide sequences of the full-length coding region of genome segment 3. The EHDV-7 strain FO-1/E/16 isolated in this study is shown in red, and other Japanese EHDV strains are shown in blue. The percentage bootstrap values calculated from 1,000 replicates are indicated around the internal nodes. The scale bar represents 0.050% sequence divergence.

Table 4. Characteristics of all the genome segments/proteins of EHDV-7 strain FO-1/E/16 and EHDV-2 strain KS-8/E/13

Segment (protein encoded)	Segment length (bp)	Size of protein (aa)	5′-Terminal hexanucleotide	5′NCR length (bp)	3′-Terminal hexanucleotide	3′NCR length (bp)
EHDV-7 strain FO-1/E/16						
Seg-1 (VP1)	3,942	1,303	GTTAAA	11	ACTTAC	22
Seg-2 (VP2)	3,002	982	GTTAAA	17	ACTTAC	36
Seg-3 (VP3)	2,768	899	GTTAAA	17	ACTTAC	51
Seg-4 (VP4)	1,983	644	GTTAAA	8	ACCTAC	40
Seg-5 (NS1)	1,770	551	GTTAAA	32	ACTTAC	82
Seg-6 (VP5)	1,641	527	GTTAAA	27	ACTTAC	30
Seg-7 (VP7)	1,162	349	GTTAAA	17	ACTTAC	95
Seg-8 (NS2)	1,186	373	GTTAAA	19	GCTTAC	45
Seg-9 (VP6)	1,074	337	GTTAAA	14	ACTTAC	46
Seg-10 (NS3)	810	228	GTTAAA	20	ACTCAC	103
EHDV-2 strain KS-8/E/13						
Seg-1 (VP1)	3,942	1,303	GTTAAA	11	ACTTAC	22
Seg-2 (VP2)	3,002	982	GTTAAA	17	ACTTAC	36
Seg-3 (VP3)	2,768	899	GTTAAA	17	ACTTAC	51
Seg-4 (VP4)	1,983	644	GTTAAA	8	ACCTAC	40
Seg-5 (NS1)	1,770	551	GTTAAA	32	ACTTAC	82
Seg-6 (VP5)	1,641	527	GTTAAA	27	ACTTAC	30
Seg-7 (VP7)	1,162	349	GTTAAA	17	ACTTAC	95
Seg-8 (NS2)	1,186	373	GTTAAA	19	ACTTAC	45
Seg-9 (VP6)	1,074	337	GTTAAA	14	ACTTAC	46
Seg-10 (NS3)	810	228	GTTAAA	20	ACTTAC	103

nucleotide of the 3′-terminal hexanucleotide was guanine in Seg-8 of the EHDV-7 strain FO-1/E/16 and adenine in the EHDV-2 strain KS-8/E/13. The two strains were closely related in the phylogenetic trees based on the full-length cDNAs of Seg-1, Seg-4, Seg-5, Seg-7, Seg-8, and Seg-9 (Figs. 4A, 4 B, 4C, 4D, 5A, and 5 B, respectively). In contrast, the two strains were sorted into different groups in the phylogenetic tree based on Seg-10 (Fig. 5C) and Seg-2 (Fig. 1). Our data suggest that these two strains might have been generated by reassortment events. In addition, a Chinese EHDV-7 strain YN09-04 was sorted into the same group as the EHDV-7 strain FO-1/E/16 in the phylogenetic trees of eight genome segments (Figs. 1–3, 4A, 4C, 4D, 5A, and 5C, respectively), but was sorted into a different group in the trees for Seg-4 and Seg-8 (Figs. 4B and 5B, respectively). Our data suggest that the two EHDV-7 strains, FO-1/E/16 and YN09-04, are closely related to each other, but their Seg-4 and Seg-8 have different origins.

Analytical specificity and sensitivity of the modified RT-PCR assay

We examined the analytical specificity of the modified RT-PCR assay to determine whether the assay could be used to detect various EHDV strains. All 21 strains of EHDV tested positive and the other arboviruses tested negative in this modified RT-PCR assay (Fig. 6). In this assay, the bands of the PCR products generated from the three EHDV strains (lanes 12, 19, and 20) were weaker than those from the other EHDV strains (Fig. 6). A possible reason for the weaker band in lane 12 is that the RNA used for the assay was obtained from blood cells of the affected cattle, as the isolation of strain EHDV-6 HG-1/E/15 was not successful [13]. Possible reasons for the weaker bands in Lanes 19 and 20 (EHDV-1 USA1955/01 New Jersey and EHDV-2 CAN1962/01 Alberta) are the lower affinities of the two strains for the primer sets.

We also determined the analytical sensitivity of the assay to determine whether the assay is sensitive enough to be used for the molecular diagnosis of EHD. We selected the EHDV-7 strain isolated in this study (strain FO-1/E/16) and the EHDV-2 strain KS-8/E/13, since EHDV-2 and EHDV-7 are major serotypes of EHDV causing illness in cattle in Japan. The detection limit of the assay was 10^3 copies per tube, which was determined using RNA synthesized with the two strains (Fig. 7).

To date, several real-time RT-PCR assays have been developed for EHDV detection; such tests are particularly useful when screening large numbers of specimens [19, 37, 38]. However, conventional PCR assays are still widely used in livestock hygiene service centers in Japan because it is challenging in terms of cost and equipment, to test diseased cattle for various pathogens using real-time PCR assays. A conventional multiplex RT-PCR assay has been widely used for the detection of arboviruses in cattle [24], which is useful for the detection of EHDV in field-collected samples. The modified RT-PCR assay developed in the present study could detect the latest Japanese EHDV-2 and EHDV-7 strains (Fig. 7) as well as various EHDV strains, including two North American strains (Fig. 6). Considering the results obtained by testing the bovine blood samples collected in the present study, we expect that the modified RT-PCR assay will be useful for the diagnosis of EHD.

Overall, our data clearly indicate that EHDV-7 infection caused fever in eight cattle bred at a farm in Fukuoka. In recent decades, epizootics of EHD in cattle have been reported in many countries in Asia and the Mediterranean basin [6, 9–11, 13, 16, 23, 29, 35, 40]. While live and inactivated EHDV-2 vaccines have been used in Japan, no other vaccines are available for the prevention of EHD in cattle. Thus, it is possible that serotypes of EHDV other than EHDV-2 will cause additional epizootics

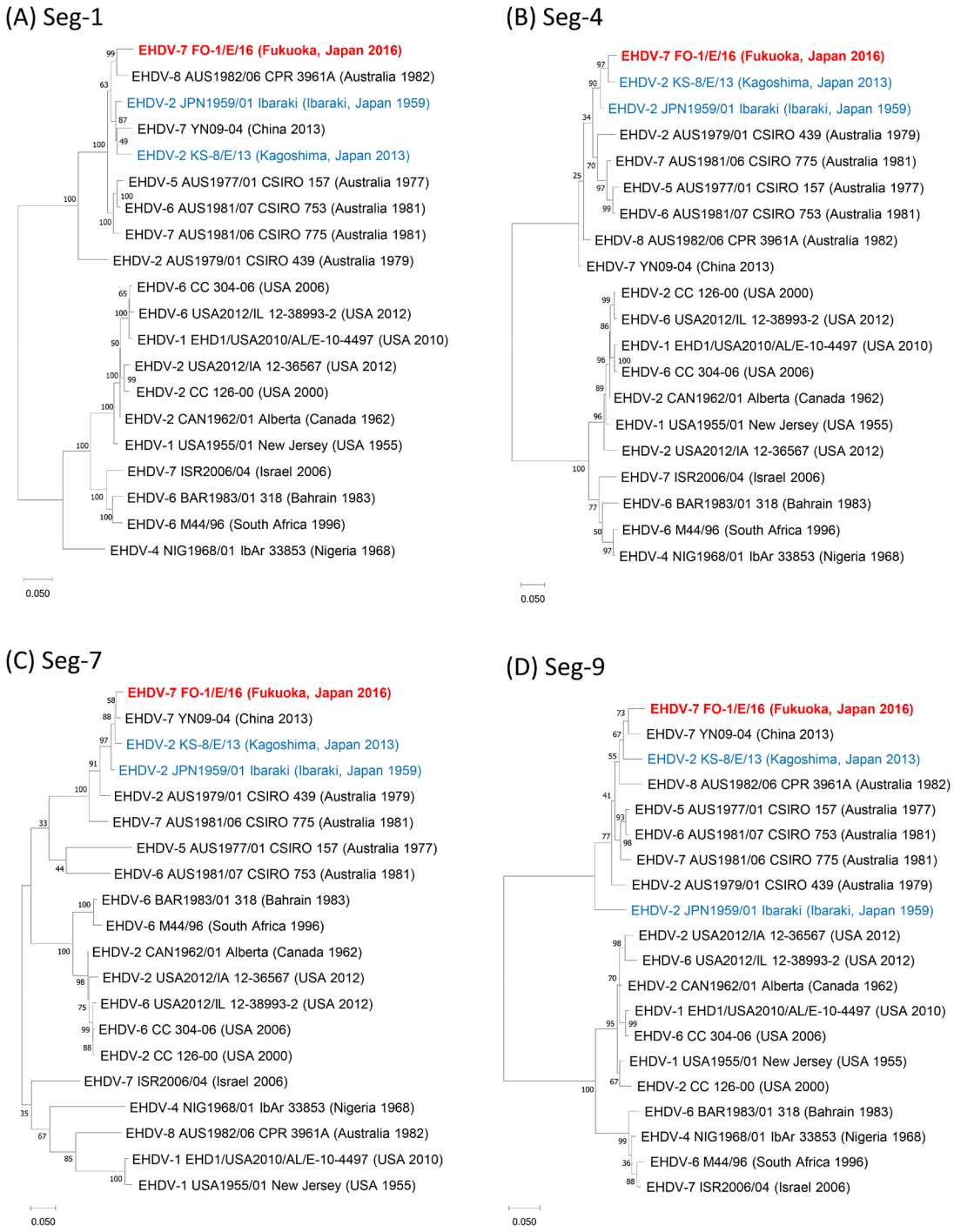

Fig. 4. Phylogenetic profile showing relationships among the epizootic hemorrhagic disease virus (EHDV) strains based on nucleotide sequences of the full-length cDNAs of genome segments 1 (panel A), 4 (panel B), 7 (panel C), and 9 (panel D). The EHDV-7 strain FO-1/E/16 isolated in this study is shown in red, and other Japanese EHDV strains are shown in blue. The percentage bootstrap values calculated from 1,000 replicates are indicated around the internal nodes. The scale bars represent 0.050% sequence divergence.

in cattle in the future. Beyond the accurate diagnosis of febrile cases and continuous monitoring for arboviral infections, the development of vaccines for serotypes of EHDV other than EHDV-2 will be necessary to reduce the economic losses caused by EHD in cattle.

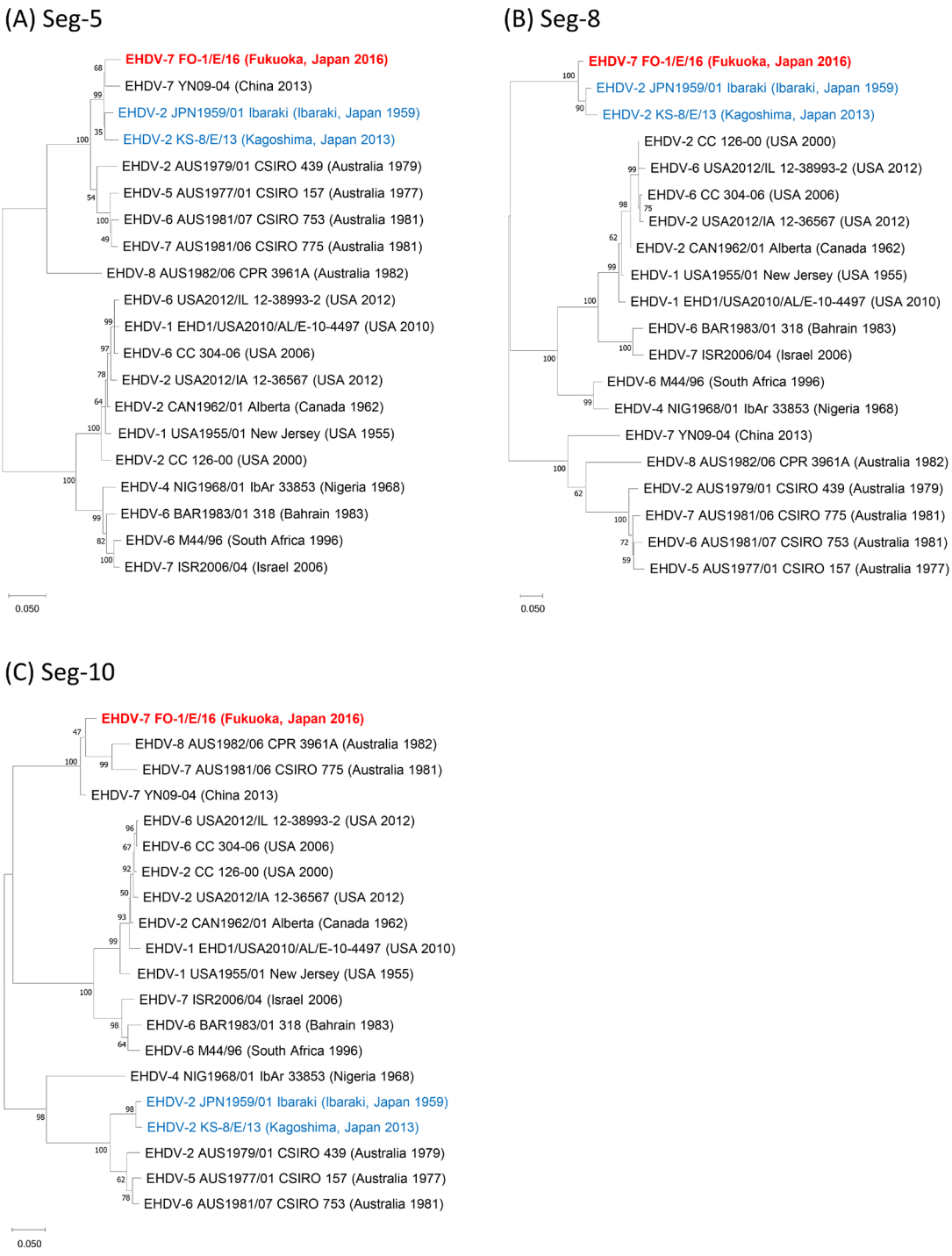

Fig. 5. Phylogenetic profile showing relationships among the epizootic hemorrhagic disease virus (EHDV) strains based on nucleotide sequences of full-length cDNAs of genome segments 5 (panel A), 8 (panel B), and 10 (panel C). The EHDV-7 strain FO-1/E/16 isolated in this study is shown in red, and other Japanese EHDV strains are shown in blue. The percentage bootstrap values calculated from 1,000 replicates are indicated around the internal nodes. The scale bars represent 0.050% sequence divergence.

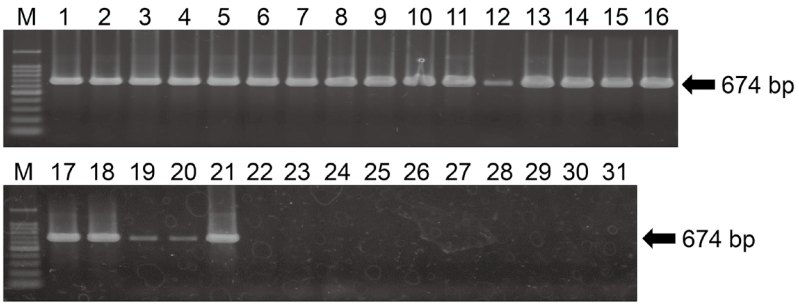

Fig. 6. Detection of epizootic hemorrhagic disease virus (EHDV) by an RT-PCR assay using a newly designed primer set. The specificity of the assay was determined with RNA templates extracted from EHDV and other viruses. Products of the RT-PCR assay were separated on agarose gels and stained with ethidium bromide. Lanes 1–4: EHDV-1 (lane 1, Kawanabe 525; lane 2, ON-1/B/01; lane 3, ON-6/E/10; lane 4, ON-1/E/13). Lanes 5–8: EHDV-2 (lane 5, NS-1/P/00; lane 6, SG-1/E/02; lane 7, KS-7/E/13; lane 8, KS-8/E/13). Lanes 9 and 10: EHDV-7 (lane 9, KSB-14/E/97; lane 10, ON-23/E/06). Lane 11: EHDV-10 ON-4/B/98. Lane 12: EHDV-6 HG-1/E/15. Lane 13: EHDV-7 ON-1/E/16. Lane 14, EHDV-2 AUS1979/05 CSIRO 439. Lane 15, EHDV-5 AUS1977/01 CSIRO 157. Lane 16, EHDV-6 AUS1981/07 CSIRO 753. Lane 17: EHDV-7 AUS1981/06 CSIRO 775. Lane 18: EHDV-8 AUS1982/06 CPR3961A. Lane 19: EHDV-1 USA1955/01 New Jersey. Lane 20 EHDV-2 CAN1962/01 Alberta. Lane 21: EHDV-2 JPN1959/01 Ibaraki. Lane 22: Akabane virus JaGAr39. Lane 23: Aino virus JaNAr28. Lane 24: Peaton virus KSB-1/P/06. Lane 25: Sathuperi virus KSB-2/C/08. Lane 26: Shamonda virus KSB-6/C/02. Lane 27: Chuzan virus K-47. Lane 28: D'Aguilar virus KSB-29/E/01. Lane 29: bluetongue virus ON89-1. Lane 30: bovine ephemeral fever virus YHL. Lane 31: RNase-free water. M: molecular mass ladder (100 bp).

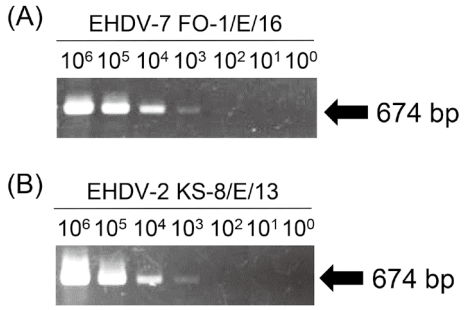

Fig. 7. Detection limits of the RT-PCR assay for two epizootic hemorrhagic disease virus (EHDV) strains: EHDV-7 FO-1/E/16 (panel A) and EHDV-2 KS-8/E/13 (panel B). A newly designed primer set, EHDV/S3/1380-1399F and EHDV/S3/2053-2034R, was used. Ten-fold serial dilutions of synthesized RNA were prepared and used as templates to determine detection limits. Products of the RT-PCR assay were separated on agarose gels and stained with ethidium bromide. M: molecular mass ladder (100 bp).

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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