

Hepatitis E Virus Variant in Farmed Mink, Denmark

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Hepatitis E virus (HEV) is a zoonotic virus for which pigs are the primary animal reservoir. To investigate whether HEV occurs in mink in Denmark, we screened feces and tissues from domestic and wild mink. Our finding of a novel HEV variant supports previous findings of HEV variants in a variety of species.

Hepatitis E virus (HEV, family *Hepeviridae*) is a main cause of acute liver inflammation in humans. It is a nonenveloped RNA virus with a positive-sense genome of ≈ 7.2 kb. In 1997, HEV was discovered in pigs (1), and several studies have since shown that HEV is endemic in pigs and that pigs probably are a major animal reservoir. HEV traditionally had been divided into 4 primary genotypes (G1–G4). G1 and G2 have been found only in humans. G3 has been found globally in a wide range of mammals, including humans, pigs, deer, rabbits, and mongooses. G4, like G3, has an animal reservoir and has been found in humans, pigs, and wild boars (2).

Along with the human and porcine variants, avian HEV (aHEV) has been characterized. It is widespread globally and has been proposed to comprise 3 genotypes (3). Since 2010, several novel HEV variants have been described in red foxes, cutthroat trout, rats, bats, and ferrets (4–8). All new variants clearly differed from HEV G1–G4, aHEV, and each other. HEV is highly prevalent among pigs in Denmark; 92% of herds are seropositive, and $\approx 50\%$ of investigated herds had pigs positive for HEV RNA (9). Because HEV is highly prevalent in swine in Denmark, animals fed offal from Danish slaughterhouses will be exposed to HEV. Production of mink fur is a major industry in Denmark, and mink are routinely fed a mixed diet, which often includes swine offal. Inappropriate heat-treated swine offal has previously been shown to be the source of swine-related influenza A virus infection in mink (10,11). Thus, we aimed to investigate whether mink in Denmark are infected with HEV G1–G4 or other HEV variants by screening fecal and tissue samples from domestic and wild mink.

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DOI: <http://dx.doi.org/10.3201/eid1912.130614>

The Study

Initially, we screened 85 fecal samples collected during 2006 through mid-2012 from farmed mink by nested PCR; a broad panel of HEV variants was detected (6). One sample was positive, and subsequent sequencing and phylogenetic analysis showed that this virus represented a new HEV variant. To screen more samples for this new virus, we developed a specific real-time reverse transcription PCR (RT-PCR) (online Technical Appendix, wwwnc.cdc.gov/EID/articlepdfs/19/12/13-0614-Techapp1.pdf). The initially tested 85 fecal samples and an additional 233 fecal samples from farmed mink, together with liver and fecal samples from 89 wild mink, were tested with this new and more sensitive assay. We identified 4 positive samples, all from farmed mink. In addition, screening with an HEV real-time RT-PCR (9) specific for G1–G4 found none positive. The HEV-infected mink were all submitted for diagnostic examination; all had histories of diarrhea in the herd. Three submissions were from herds having mink enteritis virus. Lipidosis, Aleutian mink disease virus, and catarrhal enteritis also were diagnosed in the mink (online Technical Appendix Table).

The 4 samples positive for the novel HEV variant were collected during 2008–2011 from herds across Jutland, Denmark, with a minimum distance of 80 km between the herds. The 4 PCR products obtained by the nested PCR, covering a region of 261 bp of the *RdRp* gene, were cloned and sequenced (GenBank accession nos. KC802090, KC802091, KC802092, and KC802093). The sequences were 98%–100% identical, with only 1 nonsynonymous mutation, resulting in a neutral amino acid change from isoleucine to valine (online Technical Appendix Figure). The high homology in this region is not surprising because the gene encodes the RNA polymerase. We initially tried to uncover a larger fragment by primer walking, but the limited amount of material prohibited this.

On the basis of the 261-bp fragment, we analyzed the phylogenetic relationship of this novel mink HEV variant to variants found in other animals (Figure). The mink HEV variant clustered with HEV variants found in ferrets and rats, which grouped in a separate branch that was clearly distinct from other previously described HEV variants. At nucleotide level, the mink HEV variant was $\approx 65\%$ identical to the closest classical HEV genotype (G3 and G4) and 76% and 69% identical with ferret and rat HEVs, respectively. At the amino acid level, the homologies were more pronounced, showing $\approx 87\%$ and $\approx 78\%$ identity with ferret and rat HEV variants, respectively. The grouping of the HEV reference sequences in the analysis was identical with results of previously performed phylogenetic analysis on full-length sequences (12).

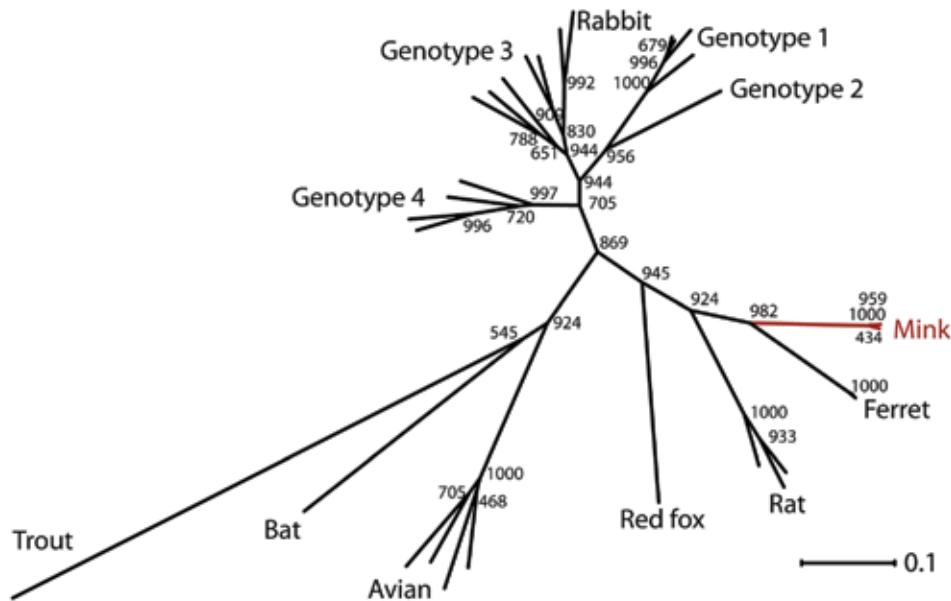


Figure. Phylogenetic tree showing the relationship between the novel mink hepatitis E virus (HEV), other HEV variants, and the 4 known HEV genotypes based on 261 bp of the *RdRp* gene. The CLC Main Workbench software (CLC bio, Aarhus, Denmark) was used for the phylogenetic analysis. Alignments were made by using MUSCLE algorithm (www.drive5.com/muscle/) and phylogenetic tree was made by using distance-based method with the neighbor-joining algorithm and bootstrap value of 1,000. Phylogenetic analysis with other methods showed similar results. Scale bar indicates nucleotide substitutions per site.

Conclusions

We detected a variant of HEV in 4 farmed mink from 4 geographically distinct locations in Denmark during a 3-year period, which indicates that the virus has been circulating among mink. Phylogenetic analysis showed that the virus was clearly distinct from, but closely related to, ferret and rat HEV variants recently reported from Germany and the United States (6,7,13).

It has not been possible to infect primates with rat or avian HEV variants (13,14). Thus, because of the phylogenetic resemblance of mink HEV with these nonzoonotic HEV variants, there are no indications that mink HEV can infect humans, although no human samples have been tested specifically for this virus. The zoonotic potential of HEV has been documented only in the case of G3 and G4, which were not found in mink. However, considering the relatively high HEV seroprevalence in humans, the possibility of other variants being zoonotic and cross-reacting with HEV G1–G4 in serologic assays cannot be ruled out.

The mink in this study were from herds that had mink enteritis virus, hepatic lipidosis, Aleutian mink disease virus, and catarrhal enteritis, all factors that could explain the conditions of the mink infected with HEV (15). However, it cannot be ruled out that the mink HEV variant contributed to the clinical signs of the mink HEV-positive animals. To determine whether the virus is indeed capable of inducing clinical signs in mink, the animals need to be experimentally infected. However, the rat and ferret HEV variants induced almost no histologic signs in rats after experimental infection, and the ferrets were described as not showing overt clinical signs (7,13). So far, only chickens infected

with aHEV and humans infected with HEV G1–G4 have been described as being clinically affected by HEV infections. The possibility exists that the HEV variants recently reported in a variety of different species, including the 1 reported here, could evolve into disease-causing pathogens in animals and possibly also humans.

Acknowledgments

We thank Mariann Chriél for supplying the samples from wild mink.

This project was funded by the Ministry of Food, Agriculture and Fisheries of Denmark (project no. 3304-FVFP-09-F-011).

Dr Krog was a PhD student at the National Veterinary Institute of Denmark, and the work presented here is part of a thesis clarifying the zoonotic aspects of hepatitis E virus in Denmark. His primary research interests include HEV infection dynamics in pigs, food safety, and viral contamination of the environment.

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Technical Appendix

nPCR

The nested PCR (nPCR) was performed as described by John et al. (1), except that in this study the primer concentrations were 10 μ M in the first round and 100 μ M in the second round of the PCR.

Real-Time RT-PCR

A real-time reverse transcription PCR (RT-PCR) that specifically detected this novel mink HEV variant was developed on the basis of the sequence obtained from the first positive sample achieved by the nPCR. The nPCR product of this positive sample was cloned in pCR4 vector using the TOPO TA cloning kit (Invitrogen, Nærum, Denmark), hereafter referred to as pMINK. The real-time RT-PCR was designed based on the Primer Probe Energy Transfer (PriProET) chemistry, which allowed a few mismatches within the probe. The primers and probe were synthesized at Eurofins MWG Operon (Ebersberg, Germany). The unlabeled forward primer Mink-Fw 5'-CCAGAATGGTGCTTCTATGGTGAT-3' had a calculated T_m of 63.6°C. The labeled reverse primer Mink-Rev 5'-FAM-AATTGTTCTGCGAGCTATCAA~~ACTC~~-3' had a calculated T_m of 62.5°C. The labeled probe Mink-Probe 5'-GCCACACCTGCCGGTCTTTGAAAACGATTT-ATTO633-3' had a calculated T_m of 75.0°C. The location of primers and probe can be seen in Technical Appendix Figure. The real-time RT-PCR was performed by using QIAGEN OneStep RT-PCR kit (QIAGEN, Hilden, Germany) in a total volume of 25 μ L, including 4 μ L extracted RNA, 100 nM Mink-Fw, 500 nM Mink-Rev, and 500 nM Mink-Probe. The assay was run on a Rotor-Gene Q real-time PCR cycler (QIAGEN) with the 72-tube rotor and channel settings at 470 nm for excitation and 660 nm for acquisition. The temperature profile was divided into 3 segments with a reverse transcription

(RT) step, a PCR cycle, and a melt analysis (MA). RT: 48°C for 30 min, 95°C for 15 min. PCR: 40 cycles of 94°C for 15 s, 55°C for 1 sec with signal acquisition, and then an additional 14 s at 55°C, 72°C for 20 s. MA: 95°C for 45 s followed by ramping the temperature from 45°C to 80°C in 1°C increments and a 7-sec halt and signal acquisition at each step. The gain-optimization was set to automatic before first acquisition on tube 2. Data analysis was performed on the Rotor-Gene Q Series Software ver. 2.0.2 (QIAGEN), and the quantitation threshold was set at 0.01. The PCR performance was tested by a standard curve performed on a 10-fold serial dilution of the constructed pMINK plasmid showing a PCR efficiency of 76% ($R^2 = 0.999$), an 8 \log_{10} detection span and an approximately detection limit at cycle threshold (Ct) 39. The melt analysis showed a T_m of $72 \pm 2^\circ\text{C}$ across the dynamic range. The sensitivity was tested on a 10-fold serial dilution of a positive mink fecal sample and compared with the nPCR. The real-time RT-PCR detected the 10^{-3} dilution, whereas the nPCR detected only the 10^{-1} dilution. All mink samples were run in duplicates with the duplicate being a 1:10 dilution of the original RNA extraction to exclude the influence of PCR inhibition.

Results

An alignment of the 4 sequenced mink HEV strains and the position of the developed mink HEV real-time PCR can be seen in the Technical Appendix Figure. A summary of the routine diagnostics analysis and the real-time RT-PCR results for the 4 positive samples are summarized in the Technical Appendix Table. One mutation was found in the probe region (Technical Appendix Figure) of the real-time RT-PCR in sequence 1119 and 574, which was consistent with the lower temperatures of the melting analysis (Technical Appendix Table).

Reference

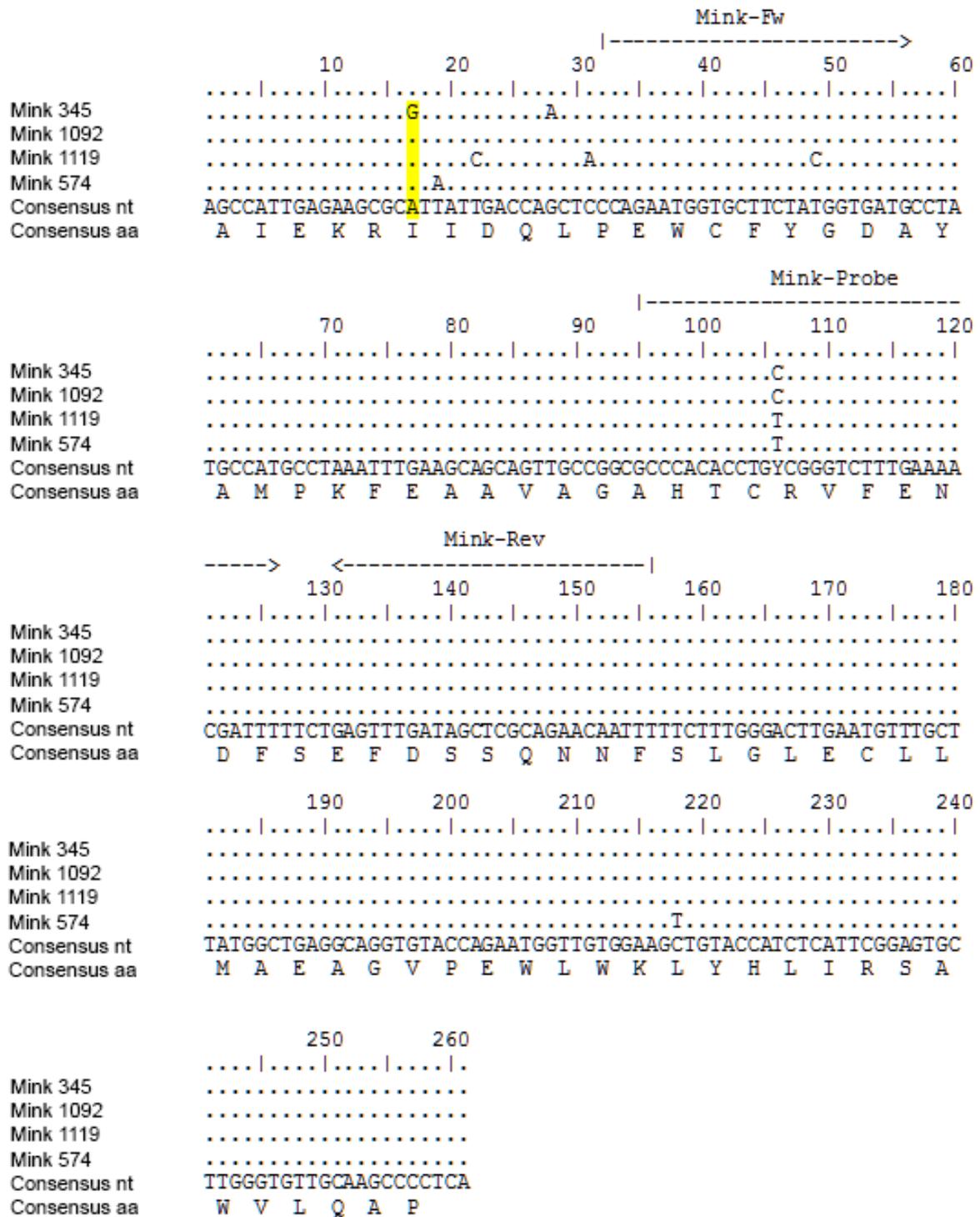
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Technical Appendix Table. Characterization of the 4 patients with hepatitis E virus–positive samples*

Sample no.	Year	Sex	Age	Pathology	Histopathology	Diagnosis	C _t	MA
345	2008	F	–	Liver: yellow, enlarged	Liver: massive diffuse lipid vacuolization	Hepatic lipidosis	25.1	73.2
					Small intestines: diffuse catarrhal enteritis	Catarrhal enteritis MeV not tested		
1092-4†	2010	F	5 mo	Liver: yellow	Liver: lipid vacuolization, stasis	Hepatic lipidosis Catarrhal enteritis	32.3	73.7
					Small intestines: catarrhal enteritis	MeV diagnosed in herd		
1092-5*	2010	M	5 mo	Liver: enlarged, hemorrhage, yellow	Liver: massive lipid vacuolization, stasis, hemorrhage	Hepatic lipidosis		
				Spleen: enlarged, hemorrhage	Small intestine: catarrhal enteritis	Catarrhal enteritis MeV diagnosed in herd		
1119	2010	M	5 mo	Enteritis	Liver: moderate to severe lipid vacuolization, stasis	Hepatic lipidosis	26.8	68.8
					Small intestine: dilatation of intestinal crypt cells, microabscesses squamous cells, shortening and fusion of intestinal villi	MeV diagnosed in subject and in herd		
574	2011	M	3 mo	No macroscopic changes	Liver: no abnormalities	Catarrhal enteritis	31.5	69.0
					Small intestine: diffuse catarrhal enteritis	MeV diagnosed in herd		

*C_t, cycle threshold; MA, melt analysis; MeV, mink enteritis virus.

†The fecal sample from 1092-4 and 1092-5 were pooled; hence only 1 C_t and MA value was recorded.



Technical Appendix Figure. Alignment of the 4 positive mink hepatitis E virus variant sequences obtained by the nested PCR. Arrows indicate the primers and probe of the real-time reverse transcription PCR. The nonsynonymous mutation is highlighted.