

**Detection and molecular characterization of cultivable  
caliciviruses from clinically normal mink and enteric  
caliciviruses associated with diarrhea in mink**

**M. Guo<sup>1</sup>, J. F. Evermann<sup>2</sup>, and L. J. Saif<sup>1</sup>**

<sup>1</sup>Food Animal Health Research Program, Department of Veterinary Preventive Medicine,  
Ohio Agricultural Research and Development Center, The Ohio State University,  
Wooster, Ohio, U.S.A.

<sup>2</sup>Washington Animal Disease Diagnostic Laboratory, College of Veterinary Medicine,  
Washington State University, Pullman, Washington, U.S.A.

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**Summary.** Enteric caliciviruses are emerging pathogens responsible for diarrhea or gastroenteritis in their respective hosts. In this report, mink enteric caliciviruses (MEC) were detected in feces from diarrheic mink by both immune electron microscopy (IEM) and RT-PCR using a broadly reactive primer pair (p289/290) targeting the highly conserved RNA polymerase regions of the enteric caliciviruses, Norwalk-like viruses (NLVs) and Sapporo-like viruses (SLVs). The MEC possess classical caliciviral morphology with typical cup-shaped depressions on the viral surface. Sequence analyses based on nucleotide and predicted amino acid (aa) sequences of the RT-PCR products indicated that MEC is most closely related genetically to SLVs of humans and animals. The MEC shared the highest aa identities (64–71%) in the RNA polymerase region with both human SLVs and the porcine enteric calicivirus (PEC) Cowden strain SLV, indicating that MEC may belong to an individual genogroup or subgroup in the SLV genus. The MEC shared only limited aa identities in the RNA polymerase region with vesiviruses (40–51%) and NLVs (29–33%). The RNA polymerase regions of the cultivable, non-enteric mink caliciviruses (MCV) were also amplified by RT-PCR using the primer pair Pol1/Pol3 based on sequences of vesiviruses, and the primer pair p289/290. Sequence analysis indicated that these MCV shared higher aa identities in the RNA polymerase region with vesiviruses (58–81%) than with SLVs (43–51%) including the MEC, lagoviruses (35–37%) and NLVs (27–35%), suggesting that they are most closely related genetically to vesiviruses. The MEC associated with diarrhea in mink are morphologically similar to but are genetically distinct from the cultivable MCV and likely represent a new member of the SLV genus.

## Introduction

Caliciviruses are small, nonenveloped viruses of 27–38 nm in diameter and possess a single-stranded, plus-sense RNA genome of 7.3–8.3 kb in length and a single structural protein of 56–70 kD. Caliciviruses in the family *Caliciviridae* are currently divided into 4 genera: 1) Vesivirus; 2) Lagovirus; 3) “Norwalk-like viruses” (NLVs); and “Sapporo-like viruses” (SLVs) [13]. *Vesiviruses* cause vesicular lesions and reproductive failure in swine and marine animals or respiratory infections in cats, dogs and calves, but were also isolated from normal animals or reptiles [3, 4, 29, 33, 36]. *Lagoviruses* cause a fatal liver necrosis and systemic hemorrhage in rabbits [25, 28]. The NLVs include a large group of viruses associated with food- and waterborne viral gastroenteritis in humans, which are subdivided into 2 distinct genogroups represented by the prototype strains, Norwalk virus (NV) and Snow Mountain virus (SMV) [6, 21]. Bovine enteric caliciviruses (BEC), Jena virus and Newbury agent-2 cause diarrhea in calves [2] and were recently characterized as NLVs [5, 24]. The SLVs including Sapporo, Manchester and Parkville viruses are mainly associated with acute, nonbacterial gastroenteritis in infants and young children worldwide [6, 17, 21, 23, 30, 31]. Porcine enteric calicivirus (PEC), Cowden strain, which causes diarrhea in pigs [10] and is the only cultivable enteric calicivirus [11, 32], has been identified as a new member in the SLV genus [14]. The caliciviruses associated with gastroenteritis in cats and chickens remain uncharacterized at the molecular level [2]. Detection of the NLVs and SLVs in feces from normal and diarrheic pigs or calves [5, 24, 34, 37, 38, 40] raises public health concern for potential cross-species transmission of enteric caliciviruses and possible cattle or swine reservoirs for enteric caliciviruses related to human caliciviruses.

The first enteric calicivirus, Norwalk virus was discovered by immune electron microscopy (IEM) of stools from diarrheic school children in Norwalk, Ohio in 1972 [21], and its genome was the first to be sequenced [18]. With applications and improvements of molecular diagnostic techniques, many newly characterized enteric caliciviruses were more recently found to be responsible for numerous outbreaks of food- and waterborne viral gastroenteritis worldwide [8, 27, 42]. By using RT-PCR with multiple primers based on highly conserved RNA polymerase sequences of many representative or prototype enteric caliciviruses, a large number of new strains of NLV or SLV were detected in stools of people involved in outbreaks of gastroenteritis [8, 19, 40] and also in feces from normal and diarrheic swine and cattle [5, 14, 24, 37, 40]. Moreover, enteric caliciviruses were also detected by RT-PCR in foods, water, sewage, fomites, etc. [12, 21, 35]. Consequently, human caliciviruses (HuCV) have emerged as the leading cause of food- and waterborne viral gastroenteritis in humans [27]. Recently, a newly-designed primer pair, p289/290 has been shown to amplify the partial RNA polymerase regions of both NLVs and SLVs [19].

Mink caliciviruses (MCV) were first isolated from normal mink on ranches with a history of hemorrhagic pneumonia (pseudomonas pneumonia), but no disease was attributed to their presence [7, 26]. The MCV were grown in Vero cells

and were antigenically distinct from VESV, SMSV and feline calicivirus (FCV) by neutralization tests [26]. Recently, coronaviruses, rotaviruses and caliciviruses were detected by electron microscopy in fecal samples collected from healthy and “sticky kits” or diarrheic young mink [20].

In this study, caliciviruses were first detected in fecal samples collected from diarrheic young mink by immune electron microscopy (IEM) using antiserum to PEC/Cowden. Then RT-PCR was performed to detect the enteric caliciviruses in mink feces by using a wide range of primer pairs for the RNA polymerase regions of SLVs and NLVs. Next, the RNA polymerase regions of 3 previously isolated, cultivable MCV strains [26] were similarly amplified by using RT-PCR. Finally, the genetic relationships between MCV and mink enteric caliciviruses (MEC) and between MEC, MCV and other human and animal caliciviruses were determined by sequence analysis and phylogenetic studies.

## Materials and methods

### *Background of the diarrhea outbreak*

The mink diarrhea outbreak occurred in early June, 1999 on a fur farm in the Northern U.S. Initially, the older mink had diarrhea which spread into the younger kits (5 weeks of age) which developed profuse diarrhea that persisted for several days. About 30 to 40% of the kits developed diarrhea. Fecal samples were collected from diarrheic kits at 2 to 6 days after the onset of diarrhea. Fecal samples collected on the same day from several diarrheic mink kits were pooled, and a total of 5 pools were collected and stored at  $-20^{\circ}\text{C}$  until use.

### *Cultivable mink caliciviruses*

The MCV isolates #9, 13, and 20 were originally isolated from nasal swabs of normal mink on ranches with hemorrhagic pneumonia (*Pseudomonas aeruginosa*) [7, 26]. The MCV were grown in Vero cells and harvested at maximum cytopathic effect within 3 to 4 days postinoculation.

### *Immune electron microscopy (IEM)*

The IEM was performed as described [34]. Mink fecal samples were diluted 1:5 in 0.01 M PBS, pH 7.2, sonicated and centrifuged at  $1,200\times g$  for 30 min. The supernatants were filtered (0.45  $\mu\text{m}$  pore-size filter), mixed with hyperimmune serum to PEC/Cowden (1:100, 1:500), hyperimmune serum to bovine coronavirus (1:100) and the dilution buffer as a control, respectively, followed by incubation at  $4^{\circ}\text{C}$  overnight. The MCV/20/80/US isolate grown in Vero cells was centrifuged at  $1,200\times g$  for 50 min to remove cellular debris and the supernatant was saved and centrifuged at  $69,020\times g$  for 2 h to pellet the virus. The virus pellet was resuspended to half the original volume in PBS and then incubated separately with the hyperimmune sera and the dilution buffer control as described above. The mixtures were then centrifuged at  $69,020\times g$  for 35 min. The pellets were washed once with distilled water and then were resuspended in distilled water and stained with 2% phosphotungstic acid, pH 7.0. Samples were examined using an electron microscope (Phillips 201, Philips-Norelco, Eindhoven, The Netherlands).

### *Extraction of viral RNA*

Viral RNA was extracted by using TRIzol LS reagent according to the instructions provided by the supplier (GibcoBRL, Grand Island, NY). Briefly, 10% suspensions of fecal samples in

0.01 M PBS, pH 7.2 were sonicated and centrifuged at  $1,200\times g$  for 30 min. The supernatant was mixed with 3 volumes of TRIzol LS reagent by vortexing and incubated at  $15\text{--}30^\circ\text{C}$  for 5 min. The mixture was mixed with 0.8 volumes of chloroform by vigorous vortexing for 1 min followed by centrifugation at  $12,000\times g$  for 15 min at  $4^\circ\text{C}$ . The viral RNA in the upper aqueous phase was precipitated with  $1\ \mu\text{l}$  of glycogen ( $20\ \mu\text{g}/\text{ml}$ ) and an equal volume of isopropanol. The RNA pellet was resuspended in  $50\ \mu\text{l}$  of DEPC-treated water and stored at  $-20^\circ\text{C}$  until use. For extraction of MCV RNA, the MCV grown in Vero cell cultures were prepared as described above.

#### *Reverse transcription-polymerase chain reaction (RT-PCR)*

Multiple primers targeting the RNA polymerase regions of caliciviruses from 3 genera in the *Caliciviridae* were used in RT-PCR for detection of potential caliciviruses in the mink fecal samples and for amplification of the target region of the 3 MCV isolates. These primers included P289/290, GLPSG1/YGDD1, JV12/JV13, NV35/NV36, NV36/NVp110, NI/NVp110, SR33/SR46, SR33/NV-3, Hel1/Hel2, Pol1/Pol2 and Pol1/Pol3 [1, 19, 22, 24, 29, 42, 43]. The primers PEC45 (<sup>4883</sup>TCTGTGGTGGCGTTAGCCTT<sup>4864</sup>) and PEC46 (<sup>4312</sup>GTGCTCTATTGCCTGGACTA<sup>4331</sup>) were designed based on the genomic sequence of PEC/Cowden [15]. We tried the primer pair, p289/290 initially in RT-PCR for amplification of the target regions of the respective caliciviruses because of its broad reactivity with NLVs and SLVs [19]. The RT-PCR was performed by using the Titan one tube RT-PCR system (Roche Molecular Biochemicals, Mannheim, Germany). Only one primer pair was used in each RT-PCR reaction. Briefly,  $5\ \mu\text{l}$  of RNA was mixed with  $1\ \mu\text{l}$  of dimethyl sulfoxide (DMSO) and 25 pmol of a reverse primer. The mixture was incubated at  $70^\circ\text{C}$  for 10 min and then chilled on ice for 2 min. To each reaction tube was added  $1\ \mu\text{l}$  of 10 mM dNTPs, 25 pmol of forward primer,  $2.5\ \mu\text{l}$  of 100 mM dithiothreitol (DTT),  $10\ \mu\text{l}$  of  $5\times$  reaction buffer,  $1.5\ \mu\text{l}$  of 25 mM  $\text{MgCl}_2$ , and  $1\ \mu\text{l}$  of enzyme mix (AMV reverse transcriptase and Taq DNA polymerase). The total  $50\ \mu\text{l}$  reaction mixtures were placed on a Perkin-Elmer GeneAmp 2400 Thermocycler (Perkin-Elmer, Norwalk, CT), equilibrated at 42 to  $50^\circ\text{C}$  and incubated for 60 min, followed by denaturation at  $94^\circ\text{C}$  for 3 min and 35 cycles of denaturation at  $94^\circ\text{C}$  for 30 sec, annealing at 42 to  $55^\circ\text{C}$  for 30–60 sec and elongation at  $72^\circ\text{C}$  for 1 min and a prolonged elongation at  $72^\circ\text{C}$  for 7 min. The PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. The PCR products of an expected size were purified by using Qiagen quick PCR purification columns (Qiagen Inc., Santa Clarita, CA), and were either directly sequenced by using an automated DNA sequencer ABI377 or cloned into a TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA) first and then sequenced.

#### *Sequence analysis*

The lasergene software (DNA Star Inc., Madison, WI) was used to analyze DNA sequences. Multiple alignments of nucleotide and predicted amino acid sequences were performed by using the University of Wisconsin Genetics Computer Group software package. To further define the genetic relationship between MEC, MCV and caliciviruses representative of each of the four genera, phylogenetic trees were generated for the predicted aa sequences of the RNA polymerase region. Sequences were aligned using CLUSTAL W [39] and the evolutionary tree was generated by using the Neighbor-Joining method [9]. The confidence values of the internal lineages within the phylogenetic trees were determined by bootstrap analysis using PHYLIP package [9]. In each bootstrap analysis, 100 bootstrap datasets were created from which trees were generated. A consensus of the bootstrapped trees was made.

*Accession number*

The partial sequences of the MCV and MEC strains have been deposited in the GenBank database.

**Results***Immune electron microscopy (IEM)*

Typical caliciviral particles were detected in all 5 pools of mink fecal specimens by IEM using hyperimmune serum to PEC/Cowden (Fig. 1, Table 1). The viruses were 30 to 35 nm in diameter and possessed cup-shaped depressions on the virion surface, which is characteristic of classical caliciviruses like vesiviruses and the SLV (Fig. 1). These viruses showed slight reactivity with hyperimmune antiserum to PEC/Cowden, but only at an antibody dilution 5–10 fold more concentrated than that reactive with PEC/Cowden. Interestingly, these viruses were morphologically indistinguishable from the cultivable MCV [7]. However, the MCV did not react with high titered hyperimmune antiserum to PEC/Cowden. The specificity of the IEM was also determined by using hyperimmune antiserum to bovine coronavirus. None of the mink fecal samples were positive for coronavirus or reactive with the antiserum to bovine coronavirus by IEM.

*RT-PCR for amplification of the viral RNA polymerase region*

By using the broadly reactive primer pair, p289/290, the expected PCR products of 331 bp were amplified by RT-PCR in all 5 pools of fecal specimens from

**Table 1.** Detection of mink enteric calicivirus (MEC) in feces of mink with diarrhea and mink calicivirus (MCV) isolates from clinically normal mink by EM, IEM and RT-PCR

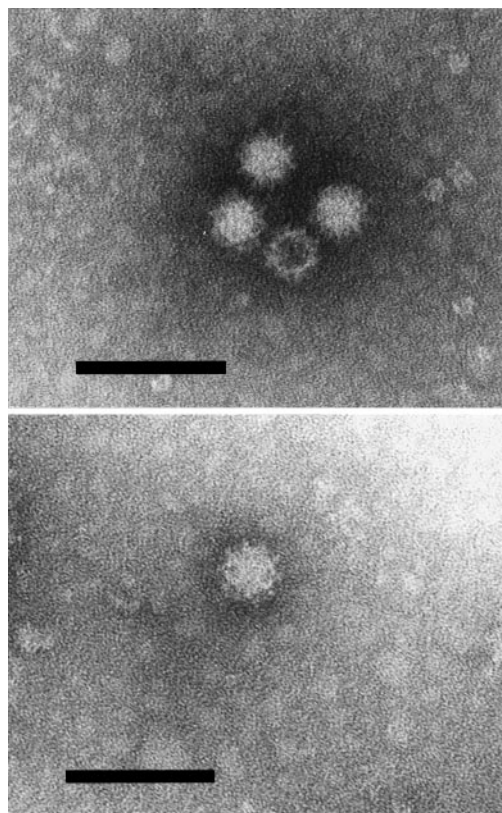
Mink sample	EM	IEM		RT-PCR with primer pair		
	MEC or MCV	Anti-PEC serum <sup>a</sup>	Anti-BCV serum <sup>b</sup>	p289/290	Pol1/Pol3	Hel1/Hel2
<i>MEC</i>						
WD1236	+	+/- <sup>c</sup>	- <sup>c</sup>	+	-	-
WD1237	+	+/-	-	+	-	-
WD1238	+	+/-	-	+	-	-
WD1239	+	+/-	-	+	-	-
WD1240	+	+/-	-	+	-	-
<i>MCV</i>						
9	+	NT <sup>d</sup>	-	+	+	+
13	+	NT	-	+	+	+
20	+	-	-	+	+	+

<sup>a</sup>Hyperimmune antiserum to PEC/Cowden

<sup>b</sup>Hyperimmune antiserum to bovine coronavirus (BCV)

<sup>c</sup> + / - = Small number of virus aggregates with antiserum to PEC compared to PBS control, - = no reaction with hyperimmune serum

<sup>d</sup>NT Not tested



**Fig. 1.** Electron micrographs of mink enteric caliciviruses (MEC) from feces (**a**) and mink calicivirus (MCV) from cell culture (**b**). Viruses in fecal or cell culture samples were incubated with hyperimmune pig anti-serum (diluted 1:100) against PEC/Cowden and a small number of aggregates were seen with MEC in **a** but not with MCV in **b**. Virus particles in the negative stain electron micrograph show the typical caliciviral morphology with distinct cup-shaped depressions on the viral surface. Bars: 100 nm

the diarrheic mink (Table 1). By setting the annealing temperature at 42 °C or 45 °C, the expected products were consistently visualized after agarose gel electrophoresis of the PCR reactions from all 5 specimens. However, if the annealing temperature was set at 50 °C or higher, no visible bands of expected products were amplified by using RT-PCR. It was noted that RT-PCR using p289/290 correlated with IEM for detecting the MEC from the mink fecal specimens (Table 1). No products with expected size were amplified from mink fecal samples by RT-PCR using primers GLPSG1/YGDD1, NV35/NV36, NV36/NVp110, NI/NVp110, SR33/SR46, SR33/NV-3, Pol1/Pol2 and Pol1/Pol3. There were also no products amplified from mink fecal specimens in RT-PCR with the PEC-specific primers PEC45/PEC46.

By using primers Pol1/Pol3, amplicons of 339 bp were amplified in RT-PCR from the three MCV isolates [9, 13, 20]. These products were smaller than those amplified for SMSV 6 and 7, VESV C52 and I55, and primate calicivirus (PCV) (419 bp) by 80 bp [29]. Sequence analysis indicated that the MCV amplicons had a deletion of 80 bp at the 3' end, which might result from the binding of Pol3 to the upper regions of the templates. With primers Pol1/Pol2, a weak product of 350 bp was amplified from MCV 9, but not from MCV 13 and 20. The amplicons with an expected size of 407 bp were also amplified in RT-PCR from the 3 MCV isolates by using primers Hel1/Hel2 based on sequences of the vesivirus helicase regions

[29]. These products were not sequenced because of the low yield. No products of expected sizes were amplified by using primers GLPSG1/YGDD1, NV35/NV36, NV36/NVp110, NI/NVp110, SR33/SR46, and PEC45/PEC46. Interestingly, amplicons of 331 bp were obtained in RT-PCR from the 3 MCV isolates by using the primer pair p289/290 at lower annealing temperature (40 to 42 °C), indicating that this primer pair was very broadly reactive with caliciviruses, including some vesiviruses.

#### *Sequence analysis*

The four RT-PCR products (331 bp) directly sequenced for MEC shared 100% nucleotide sequence identities with each other, suggesting the same original source for viruses in the mink fecal specimens. The predicted amino acid sequences of the amplicons contained the GLPSG motifs characteristic of the RNA polymerase regions of caliciviruses and viruses in the *Picornaviridae* superfamily. The amplicons shared only limited nucleotide and aa sequence identities with the RNA polymerase regions of vesiviruses (34–43%, 46–54%), lagoviruses (34–37%, 40%) and NLVs (27–38%, 29–33%) including the BEC, Newbury agent-2 and Jena virus (partial data shown in Table 2), respectively. However, they shared the highest nucleotide and aa identities with the RNA polymerase regions of SLVs (54–61%, 64–70%, respectively). The MEC shared 68.5% amino acid identity in the RNA polymerase region with PEC/Cowden, newly characterized as a SLV of animal origin.

The amplicons produced with primer pairs p289/290 and Pol1/Pol3 from the 3 MCV isolates were sequenced directly and the overlapping regions were aligned to generate longer fragments (464 bp). The assembled fragments contained GLPSG and YGDD motifs characteristic of the RNA polymerases of caliciviruses. The MCV 20 shared 96.1% nucleotide and aa sequence identities in the partial RNA polymerase region with both MCV 9 and 13, whereas the MCV 9 shared 98.1% nucleotide and 100% aa sequence identities in the same region with the MCV 13 (data not shown). The 3 MCV isolates shared only limited nucleotide and aa identities in the RNA polymerase region with SLVs (39–44%, 43–51%), RHDV (33%, 35–37%) and NLVs (27–34%, 27–35%), respectively, but shared much higher nucleotide and aa identities with vesiviruses (50–65%, 58–81%, respectively) (partial data shown in Table 2). The MCV shared higher aa identity in the RNA polymerase region with canine calicivirus (81%) than with VESV/SMSV (62–69%) and FCV (58–64%) in the vesivirus genus. Comparatively, the MCV shared lower nucleotide and aa identities (37–39%, 47–50%, respectively) in the RNA polymerase region with the MEC identified in fecal samples of the diarrheic mink.

#### *Phylogenetic tree of the RNA polymerase regions*

The phylogenetic tree generated for the RNA polymerase regions of MEC and MCV, using the predicted aa sequences (Fig. 2) was similar to that generated for the same region by using nucleotide sequences (data not shown). The evolutionary

**Table 2.** Percent amino acid sequence identity in the RNA polymerase region between MEC, MCV and PEC, SLVs, NLVs and vesiviruses

	MV	SV	Hou90	Lon92	PEC	MEC	FCV	SMSV1	PCV	CaCV	RHDV	NV	JV	NA-2	SMV	HV	Sw/NLV/48
<i>SLV</i>																	
MEC	70.5	69.5	64.8	65.7	68.6	51.4	46.7	45.7	45.7	45.7	40.4	32.7	31.7	31.7	32.7	30.7	29.2
<i>Vesivirus</i>																	
MCV #9	50.5	50.5	44.8	48.6	44.8	49.5	66.7	67.6	81.0	36.5	29.7	32.7	32.7	32.7	30.7	30.7	29.2
MCV #13	50.5	50.5	44.8	48.6	44.8	49.5	66.7	67.6	81.0	36.5	29.7	32.7	32.7	32.7	30.7	30.7	29.2
MCV #20	47.6	47.6	42.9	46.7	42.9	46.7	61.9	62.9	76.2	34.6	26.7	29.7	29.7	29.7	27.7	27.7	22.9

Abbreviations for virus strains are as follows: SLV genus: SV-Sapporo, MV-Manchester, Hou90-Houston/90/US, Lon92-London/92/UK, PEC-porcine enteric calicivirus Cowden strain, MEC-mink enteric caliciviruses; NLV genus: NV-Norwalk, JV-Jena virus, NA-2-Newbury agent-2, SMV-Snow Mountain virus, HV-Hawaii, Sw/NLV/48-Swine Norwalk-like virus detected in Japan; *Vesivirus*: SMSV-San Miguel sea lion virus serotype sp 1, FCV-Feline calicivirus, PCV-primate calicivirus, CaCV-Canine calicivirus, MCV-mink caliciviruses; *Lagovirus*: RHDV-rabbit hemorrhagic disease virus. The highly conserved sequences from aa 1524 to 1624 (from TAWDS to YGDD motif) of ORF1 (NV numbering) were aligned for RNA polymerase with similar sequences of indicated caliciviruses



tree indicates that MEC is more closely related genetically to the SLVs than to other human and animal caliciviruses. The MEC fell into the PEC/Cowden branch, but showed genetic diversity from PEC/Cowden and human SLVs. The MCV isolates 9, 13, and 20 are most closely related genetically to vesiviruses. MCV fell into the canine calicivirus (CaCV) branch, but remained genetically distinct from VESV/SMSV, FCV and even CaCV based on their aa sequence identities in the RNA polymerase region (Table 2). All the known calicivirus reference strains were segregated or differentiated into the respective genogroups or genera in the *Caliciviridae* family, and the evolutionary tree created for the sequences of the viruses in the individual genus is concordant with that in Fig. 2, which indicated 4 distinct major branches representing 4 different genera in the *Caliciviridae* family. The bootstrap values ranged from 46 to 100%, and for the major nodes that differentiated the viruses into separate genotypes the bootstrap values were often up to 95–100%.

### Discussion

A recent report suggests that HuCV (NLVs and SLVs) cause 23 million cases of foodborne illnesses in the U.S. annually, accounting for 67% of the cases caused by foodborne pathogens in the U.S. and 33% of annual hospitalizations due to foodborne illnesses [27]. The recognition of HuCV as an important foodborne pathogen relies largely on the development and improvement of molecular assays for detection of HuCV in stools and other specimens. Because of the great genetic diversity among caliciviruses, multiple primer pairs usually need to be used in RT-PCR for sample examination. A broadly reactive primer pair, p289/290 based on the RNA polymerase sequences of 25 prototype and currently circulating HuCV strains in the NLV and SLV genera was able to detect more viruses in either the NLV or SLV genera than previously designed primers [19].

In this study, calicivirus particles were detected by IEM in all 5 pooled fecal samples collected from the diarrheic mink kits during an outbreak of diarrhea in a mink ranch. The viruses possessed the typical classical calicivirus morphology characterized by cup-shaped depressions on the virion surface. No coronaviruses or other virus-like particles were observed, suggesting that the “classical calicivirus” detected in mink feces might be associated with the diarrhea in kits. By using RT-PCR with the primer pair p289/290, we obtained products of 331 bp (expected size for SLVs) from all 5 mink fecal samples. These products contained the GLPSG motif and shared the highest aa identities (60–70%) with the RNA polymerase regions of SLVs, suggesting that MEC is a new member in the SLV genus.

MEC was morphologically indistinguishable from the MCV originally isolated from normal mink [7, 26]. The cultivable MCV were antigenically distinct from FCV and the multiple serotypes of VESV and SMSV by virus neutralization tests [26]. Thus it was of interest to determine the genetic relationships between MEC and MCV. We used primer pair Pol1/Pol3 based on the RNA polymerase sequences of VESV and SMSV [29] and successfully amplified products of 339 bp



from all 3 MCV isolates by using RT-PCR. These products were smaller than the expected ones for some VESV and SMSV [29], because of the 3' deletion (80 bp) that may result from unexpected binding of Pol3 to the upper regions of the templates. By using the primers p289/290, we also obtained the expected products from all 3 MCV isolates. This is further evidence of the extremely broad reactivity of p289/290 to detect not only the NLVs and SLVs, but also some vesiviruses such as the MCV. The enteric caliciviruses associated with diarrhea in pigs, cattle, chicken and dogs were initially detected by EM or IEM [2, 34, 38], but this method is cumbersome, insensitive and time-consuming to use for large numbers of fecal samples and hence not practical for large scale epidemiologic surveys. Because only a few enteric caliciviruses of animal origin are characterized and limited sequence data are available, the calicivirus broadly reactive primers p289/290 can be used for the clinical diagnosis of enteric caliciviral infections in humans and animals and for environmental monitoring.

The amplicons obtained with p289/290 from MCV overlapped the 5' end of the products amplified with primer pair Pol1/Pol3 and sequence assembly generated a fragment of 465 bp. The assembled fragments contained the GLPSG and YGDD motifs characteristic of the calicivirus RNA polymerases and shared the highest aa sequence identities with the RNA polymerase regions of vesiviruses (58–81%). The 3 MCV isolates shared very high nucleotide and aa sequence identities (96–100%) with each other, which correlates with their close antigenic relatedness determined by virus neutralization tests [26]. Similarly, the genetic divergence between MCV and VESV, SMSV and FCV correlated with their antigenic distinctiveness. Usually, the caliciviral RNA polymerase region is more conserved than the capsid region which determines the viral antigenic identity and serotype. It was proposed that viruses with less than 80% aa identity in the complete capsid



**Fig. 2.** Phylogenetic tree generated for the sequences in the RNA polymerase region of caliciviruses. Amino acid sequence alignment was generated from the SKWDS sequence to the YGDD motif of the RNA polymerase. Calicivirus sequences used in the alignment were retrieved from GenBank. Strain names and abbreviations (GenBank accession numbers) are as follows: SLV: SV-Sapporo (S77903), MV-Manchester (X86559), PV-Parkville (U73124), Hou86-HuCV Houston/86 (U95643), Hou90-Houston/90 (U95644), Lon92-London/92 (U67857), PEC/Cowden (AF182670), MEC-mink enteric calicivirus (XXXX); *Vesiviruses*: FCV-feline calicivirus (M86379), SMSV-1-San Miguel sea lion virus serotypes 1 (U15301), SMSV-4 (U15302), SMSV-5 (U18731), SMSV-6 (U18732), SMSV-13 (U18734), SMSV-14 (U18735), VESV-vesicular exanthema of swine virus, A-48 (U18737), I-55 (U18740), BCV-bovine calicivirus (U18741), PCV-primate calicivirus (AF091736), MCV/9/80/US-mink calicivirus, MCV/13/80/US, MCV/20/US; *Lagovirus*: RHDV-rabbit hemorrhagic disease virus (M67473); NLV: NV-Norwalk (87661), DSV-Desert Shield (U04469), SHV-Southampton (L07418), JV-Jena virus (bovine 117/80/FRG) (AJ011099), NA-2-Newbury agent-2, Snow Mountain virus (L23831), Toronto virus (U02030), Mexico virus (U22498), Hawaii virus (U07611), Lordsdale virus (86557), Bristol virus (X76716), Oth-25 virus (L23830), Sw/NLV/43/98/-swine calicivirus (AB009415) detected in Japan, Bo/NLV/176-Norwalk-like virus detected in calves in the Netherlands in 1998 (AF194183), Sw/NLV/34-Norwalk-like virus detected in pigs in the Netherlands in 1998 (AF194184)

gene may represent distinct antigenic types or serotypes [16]. Our findings provide a further genetic basis supporting the antigenic distinction between MCV and other vesiviruses. The MCV were the most closely related genetically to CaCV, a recently identified vesivirus genetically different from SMSV/VESV and FCV in the *Vesivirus* genus [33]. However, the MCV and CaCV may comprise two independent genogroups distinct from VESV/SMSV and FCV in this genus because the MCV shared only 81% aa identity with CaCV even in the highly conserved RNA polymerase region [16, 17].

MEC are morphologically similar to, but genetically distinct from MCV based on partial RNA polymerase sequence analyses, which was confirmed by phylogenetic studies of the predicted aa sequences. MEC was most closely related genetically to SLVs and may be a new member of this genus. From the phylogenetic tree, MEC was assigned to the same branch as PEC/Cowden, but both of them shared only 68.6% aa identity in the RNA polymerase region with each other. Thus MEC may comprise an independent genogroup distinct from Sapporo/Manchester virus, Houston/90/Parkville virus, London/92 and PEC/Cowden in the SLV genus [17].

Enteric caliciviruses cause diarrhea in many animal species [2], but only a few have been characterized molecularly, including PEC/Cowden, BEC Newbury agent-2 and Jena virus [5, 14, 24]. To our knowledge, this is the first report of the molecular characterization of a MEC associated with diarrhea in mink. Interestingly, NLV genes were detected in cecal contents of slaughtered pigs in Japan [37] and in pooled fecal specimens of farm animals (veal calves and pigs) in the Netherlands [40], raising public health concerns for potential zoonotic transmission of enteric caliciviruses of animal origin. The MEC is closely related genetically to SLVs, but its source is unknown. In this mink ranch, the mink affected by the diarrhea outbreak were fed raw kidneys and livers from pigs and cattle. It is unknown if such unprocessed animal by-products were contaminated by enteric caliciviruses from swine or cattle and thus were the source of infection for mink. Further studies are needed to clarify if such MEC infect pigs or calves and if these animals and mink are possible reservoirs for enteric caliciviruses potentially transmissible to humans. The origin and the lack of pathogenicity of MCV for mink is of additional interest. It was suggested that MCV from normal mink might originate from marine animals because the affected mink were commonly fed marine by-products and the MCV infection was widespread in mink based on serology [26, 36]. However, the MCV were genetically (this report) and antigenically distinct from the SMSV/VESV [26].

In summary, the MEC associated with mink diarrhea was identified as a potential new member of the SLV genus, and it was genetically distinct from the MCV that were characterized as potential new members of the *Vesivirus* genus. Thus the enteric and non-enteric caliciviruses from mink were genetically distinct from each other. The primer pair, p289/290 proved to be very broadly reactive, not only with NLVs and SLVs including the MEC associated with mink diarrhea, but also with the MCV of the *Vesivirus* genus, indicating that this primer pair can be used for clinical diagnosis and epidemiological investigations.

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Authors' address: Dr. L. J. Saif, Food Animal Health Research Program, Department of Veterinary Preventive Medicine, Ohio Agricultural Research and Development Center, The Ohio State University, 1680 Madison Avenue, Wooster, OH 44691, U.S.A.

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