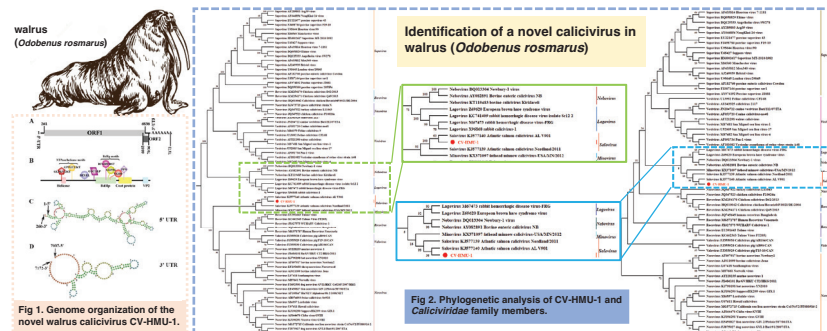


ORIGINAL ARTICLE



Identification of a Novel Calicivirus in Walrus (*Odobenus Rosmarus*)

Graphical abstract



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Highlights

- This study describes CV-HMU-1, the first *Salovirus* identified in walruses, which features two conserved open reading frames (ORFs) with distinct *Salovirus* motifs and 75.68% amino acid identity with respect to fish caliciviruses. Our findings expand virological classification and indicate evolutionary links between calicivirus transmission in terrestrial and marine mammal hosts; this important information on calicivirus genetic diversity may aid in the prevention of viral diseases.

Brief statement

We identified a novel calicivirus (CV-HMU-1) in walruses with diarrhea through metagenomics. Phylogenetic analysis (VP1/VP2) suggested that CV-HMU-1 is a novel *Salovirus* species within the *Caliciviridae*.

Identification of a Novel Calicivirus in Walrus (*Odobenus Rosmarus*)

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Abstract

Background: Caliciviruses are associated with several disease symptoms, such as diarrhea, respiratory infections, vesicular lesions, reproductive failure, and fatal hemorrhagic disease in animals. Novel disease-causing caliciviruses continue to be discovered in mammals.

Methods: Through a metagenomics approach, we identified and genetically characterized a novel calicivirus in swab samples from the noses, throats, and anuses of walruses (*Odobenus rosmarus*) with diarrhea in Qingdao Polar Haichang Ocean Park, China, between April and December 2018.

Results: The genomic sequence of the novel walrus calicivirus CV-HMU-1 was 7,172 nt long and included two complete open reading frames (ORFs) encoding classical calicivirus NS protein amino acid motifs. Phylogenetic analysis based on amino acid sequences including viral protein 1 (VP1) and viral protein 2 (VP2) from representatives of 11 known genera confirmed that CV-HMU-1 is a new member of the *Salovirus* genus of the *Caliciviridae* family. The non-structural (NS) CV-HMU-1 shared 75.68% and 64.68% amino acid identity with *Atlantic salmon calicivirus* and *Ameiurus nebulosus calicivirus 1*, respectively, which are also *Salovirus* species. However, further research is needed to verify the direct link between CV-HMU-1 and diarrhea.

Conclusions: CV-HMU-1 is proposed as a novel *Caliciviridae* species. Our findings not only expand virological classification but also indicate evolutionary links in calicivirus transmission between terrestrial and marine mammal hosts.

Keywords: calicivirus, *Salovirus*, diarrheal walrus, metagenomics approach

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BACKGROUND

Caliciviridae is a family of viruses infecting fish and marine and terrestrial animals, and causing diverse diseases, such as non-bacterial gastroenteritis, respiratory infections, vesicular lesions, reproductive failure, and fatal hemorrhagic disease [1]. The *Caliciviridae* comprise 11 genera: *Norovirus* (NoV), *Sapovirus* (SaV), *Lagovirus*, *Vesivirus*, *Nebovirus*, *Recovirus*, *Valovirus*, *Bavovirus*, *Nacovirus*, *Minovirus*, and *Salovirus*. Both NoVs and SaVs infect humans of all ages

and cause epidemic acute gastroenteritis, which can occur sporadically or as outbreaks. Human NoVs (HuNoV) can replicate in human B cells that express histo-blood group antigen (HBGA), synthesize H-type HBGA [2], or propagate in human enterocytes [3]. SaVs are transmitted via the fecal-oral route, and can infect animals including pigs, minks, dogs, sea lions, and bats; however, they cannot bind HBGAs [4]. Rabbit hemorrhagic disease virus, the main *Lagovirus* representative,

can invade multiple organs, such as the liver, spleen, and blood mononuclear cells, and cause disseminated intravascular coagulation, thus leading to multiple organ hemorrhage and necrosis [5]. Since *Vesivirus* was first isolated from California sea lions in 1972, several species of pinnipeds and cetaceans have exhibited susceptibility to calicivirus infections. These viruses cause vesicular lesions in flippers, abortion, and premature birth in pinnipeds. For example, the San Miguel sea lion virus causes exanthemas and diseases of the reproductive system [6]. *Nebovirus* was established as a novel genus within *Caliciviridae* in 2006, with Newbury agent 1 (Bo/Newbury1/1976/UK) designated as the exemplar isolate of the species, representing a cattle-pathogenic enteric virus [7]. Bovine NoV and *Nebovirus* infections in calves are widespread in China, the USA, Brazil, Turkey, Iran, and South Korea.

Caliciviruses have an icosahedral protein capsid with 7.3–8.5 kb RNA. Their genome contains VPg-linked positive-sense RNA, open reading frames (ORFs), and UTRs [8]. ORF1 encodes a polyprotein in various caliciviruses, whereas NoVs contain viral protein 1 (VP1) and viral protein 2 (VP2) sequences in ORFs 2 and 3, and *Vesivirus* ORF2 produces VP1 precursor protein, which is cleaved into mature VP1 and LC, and subsequently promotes calicivirus spread [9,10]. Bioassays are considered the first line of defense in the prevention and control of infectious diseases [11].

Marine mammals are crucial apex predators with essential ecological roles in marine ecosystems [12–14]. The global focus on marine mammal populations underscores their critical health status: these populations are increasingly threatened by overfishing, habitat destruction, marine pollution, and pathogenic infections [15,16]. Recent studies have indicated that marine mammals in human care exhibit longer lifespans than their wild counterparts [17,18]. Given the substantial number of marine mammals in human care, understanding the viruses that they carry is essential for maintaining their health and well-being, both in captivity and in the wild, and for preventing zoonotic diseases [13,17]. The rapid development of omics technology has importantly enabled viral evolution, adaptation, and diseases to be revealed [19–22]. We have previously discovered one novel astrovirus [23], one novel picornavirus [24], and two novel papillomaviruses [25] in marine mammals under human care. In this study, we used a metagenomics approach to detect new viruses in marine mammals under human care, aiming to explore the diversity of viruses carried by these mammals and to further elucidate their potential pathogenicity, and risks of cross-species transmission, through evolutionary and genomic sequence feature analyses.

METHODS

Samples and metagenomic sequencing

We collected 18 swab samples from the nose, throat, and anus in *O. rosmarus* with diarrhea, which were housed in aquaria in Qingdao Polar Haichang Ocean Park, China,

between April and December 2018. The samples were immediately immersed in a maintenance medium in virus-sampling tubes (Yocon, China), transported to the laboratory within 24 h via dry-ice cold chain transportation, and stored at -80°C .

All samples were combined into one pooled sample, mixed, and centrifuged at 12,000 rpm for 5 min at 4°C . The supernatant was filtered through $0.45\text{ }\mu\text{m}$ filter units (Millipore, USA). Subsequently, the viral genome of the mixed pool was extracted with a QIAamp viral RNA kit (Qiagen, Germany), and complementary DNA (cDNA) was synthesized as viral nucleic acid libraries with a Superscript III cDNA synthesis kit and random hexamers (Invitrogen, USA). Amplified viral nucleic acid libraries were analyzed on an Illumina HiSeq2500 sequencer. The sequence data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (accession number: PRJNA650224).

Taxonomic assignment

Taxonomic assignments were based on sequence similarity. Briefly, each read was evaluated for viral origin through alignment against the NCBI non-redundant nucleotide and protein (NR) databases with Blastn and Blastx ($-e$: expected value $< 10^{-5}$, $-F$: filter query sequence, default = T). Taxonomies of aligned reads with the best BLAST scores (E -value $< 10^{-5}$) from all lanes were analyzed and exported with Meta Genome Analyzer (MEGA) 6 [26].

Sequence analysis and assembly

Molecular information from the metagenomic analyses was used to classify the sequence reads into virus families or genera via MEGA 6. Representative reads for the novel calicivirus were selected for genome sequencing and read-based polymerase chain reaction (PCR) to identify partial genomes. The general structural information of the reads was obtained with Blastx and SeqMan, and the sequence map contained several gaps. We subsequently acquired gap information with PCR and Sanger sequencing. Viral RNA was isolated from individual samples with a QIAamp Viral RNA Mini Kit (Qiagen, USA), and cDNA was generated with random primers and Superscript III Reverse Transcriptase (Invitrogen, USA). The remaining genomic sequences were determined through genome walking and 5'- and 3'- rapid amplification of cDNA ends with a RACE DNA Purification System (Invitrogen, USA) and 3'-Full RACE Core Set with PrimeScriptTM RTase (TaKaRa, Japan), according to the manufacturers' instructions.

Phylogenetic analyses

Maximum likelihood phylogenetic trees were constructed with MEGA version X on the basis of the VP1 and VP2 partial amino acid sequences of members of the 11 genera in the *Caliciviridae* family. The evolutionary tree based on VP1 consisted of partial VP1 amino acid sequences of 74 representative caliciviruses, and the evolutionary

tree based on VP2 consisted of partial VP2 amino acid sequences of 64 representative caliciviruses. Selected sequences were obtained from GenBank and the present study, and a maximum likelihood analysis was performed with 1000 bootstrap replicates.

Prevalence of calicivirus infection in 18 swab samples from *O. rosmarus*

Specific nested PCR primers targeting the non-structural (NS) gene of the novel calicivirus CV-HMU-1 were designed to screen the *O. rosmarus* swab samples from the nose, throat, and anus. Viral RNA was isolated with a QIAamp Viral RNA mini kit (Qiagen, USA), cDNA was generated with random primers and Superscript III Reverse Transcriptase (Invitrogen, USA), and PCR was performed with 2× Taq PCR Mastermix (Tiangen, China). First, 5 µL of the first-round PCR product was used as the template for the second round of PCR. Equal amounts of ddH₂O were added as negative controls in both PCR rounds. The thermal cycling conditions for both rounds were 94 °C for 5 min; 35 cycles at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s; and a final elongation step at 72 °C for 10 min. Finally, the PCR products were analyzed via 1.0% agarose gel electrophoresis and ultraviolet imaging.

Genome annotation

The complete genomic sequence of the novel virus obtained via end amplification was submitted to the Conserved Domain Search Service (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) and InterProScan 5 (<http://www.ebi.ac.uk/services/proteins>) to predict the conserved protein families and domains. Classic important protein motifs were deduced by comparison of the sequences with those of other caliciviruses. Routine sequence alignment was performed with Clustal Omega (<http://www.ebi.ac.uk/Tools/>).

RESULTS

Metagenomic sequence data and read assembly

A total of 238 MB nucleotide data (449,904 valid reads, 100 bp in length) were obtained from 18 nose, throat, and anus swab samples from *O. rosmarus* with diarrhea. Reads classified as cellular organisms (bacteria, archaea, and eukaryotes) and those with no significant similarities to any amino acid sequence in the NR database were removed. The remaining 124,352 reads, accounting for approximately 27.64% of all sequence reads, were best matched with viral proteins from the NR database.

A total of 348 *Caliciviridae*-associated reads (approximately 0.28% of all viral reads) were assembled into four contigs with three gaps among them, through SeqMan analysis.

Genomic characterization of the novel calicivirus

Through read-based PCR and 5C- and 3C-rapid amplification of cDNA ends, a 7,172 bp genomic sequence known

as CV-HMU-1 (GenBank accession No. OQ110727) was assembled. The polyprotein amino acid sequence identity of CV-HMU-1 with respect to *Atlantic salmon calicivirus* (ASCV) (AHX24377.1), Wuhan carp calicivirus 2 (AVM87200.1), and Guangdong pseudohemiculter dispar calicivirus (AVM87230.1) was 77.36%, 71.43%, and 66.33%, respectively. CV-HMU-1 contained two complete ORFs (Fig 1A and B). Fig 1C and D display the predicted secondary structure. The 5'-UTR was 260 nt, and the 3'-UTR was 136 nt. The poly(A) tail of CV-HMU-1 was similar to those of other caliciviruses.

ORF1 was 6,576 nt long (261–6836) and encoded a 2,192 aa polyprotein, which was initiated and terminated by ATG and TAG codons, respectively. Several important calicivirus NS protein amino acid motifs were identified in the protein sequences translated from ORF1. These motifs included two GXPGXGKT NTPase/helicase motifs [27]: GLPGVGKT at position 1365–1389 and GPPGCGKT at position 2040–2064. In addition, a DEYEL VPg motif was found at position 2868–2883, a GDCGLP 3C-like protease motif was found at position 3489–3507, and a VP1 motif PPN was found at position 5445–5454. Three RNA-dependent RNA polymerase (RdRp) motifs, YGDD, GLPSG, and DYXXWDST (or DFGRWDST), were located at positions 4548–4560, 4416–4431, and 4260–4284, respectively [28,29]. The conserved protein domains of ORF1 are shown in sequence for one RNA helicase (at position 1353–1652), one P-loop NTPases (at position 1944–2081), an RdRp (at position 4011–4922), and a calicivirus coat protein (at position 5085–5948), which spanned the conserved amino acid motif regions (Fig 1B). Therefore, these findings indicated that ORF1 included NTPase/helicase, VPg, protease, RdRp, and VP1 sequences. The NS of CV-HMU-1 shared 75.68% and 64.68% amino acid identity with ASCV [27] and *Ameiurus nebulosus calicivirus 1* [30], respectively.

ORF2 was 375 nt long (6662–7036) and encoded a 125 aa viral protein referred to as putative minor structural protein (VP2), which showed 82.14% amino acid sequence identity with respect to ASCV (YP 009026988.1), according to Blastx. ORF2 was initiated and terminated by an ATG and TAA codon, respectively. An overlapping fragment with 175 nt was present between ORF1 and ORF2 (Fig 1).

Prevalence of calicivirus infection among *O. rosmarus* samples

All 18 samples from *O. rosmarus* with diarrhea were screened via PCR with gene-specific nested primers targeting the NS region of the novel calicivirus. Only one anal swab sample tested positive for the novel calicivirus. The positivity rate was 5.6%.

Phylogenetic analyses

Phylogenetic analysis was based on amino acid sequence regions of representative caliciviruses from the 11 known genera. The results confirmed that the walrus calicivirus

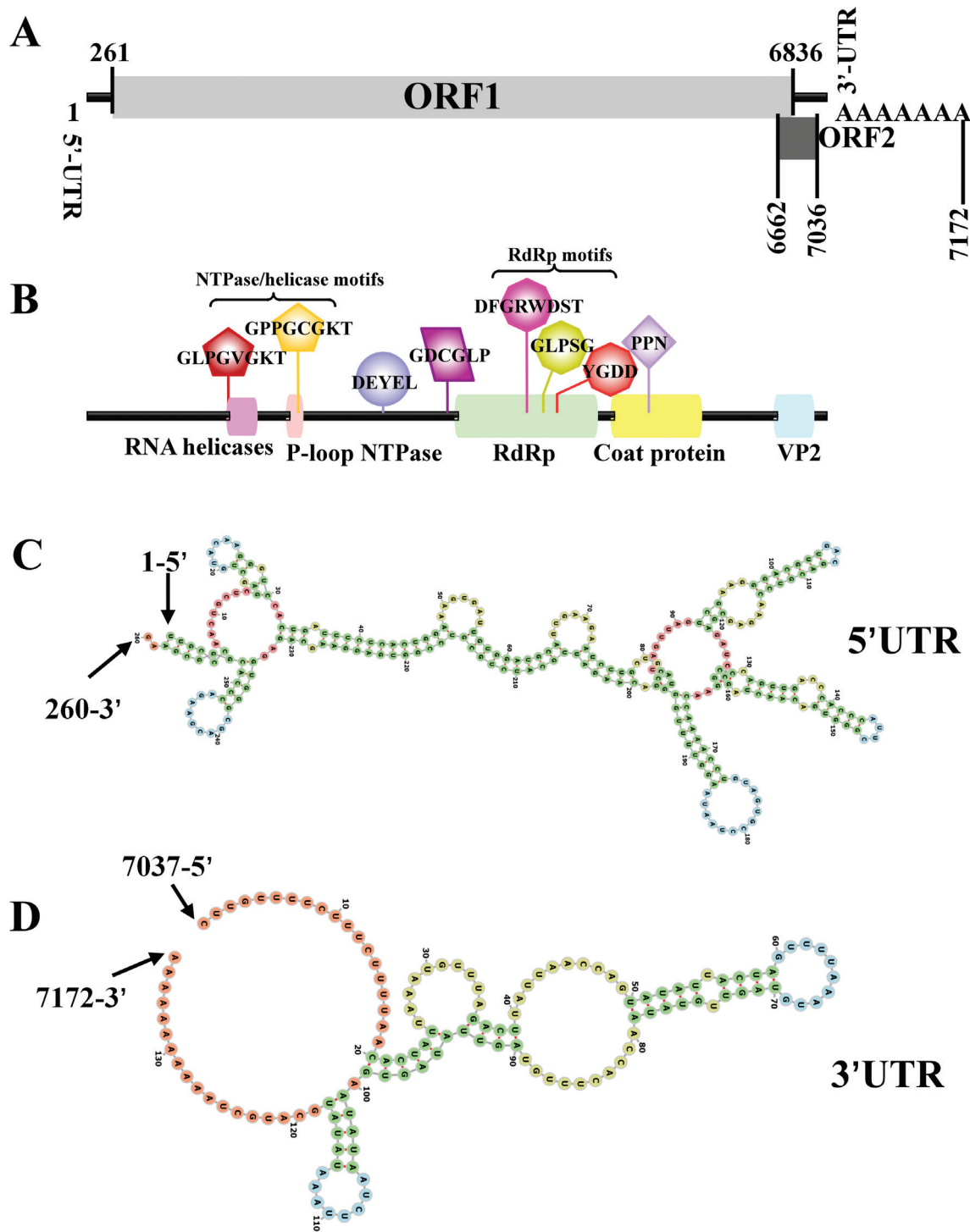


FIGURE 1 | Genomic organization of the novel walrus calicivirus CV-HMU-1.

A) The walrus calicivirus CV-HMU-1 has two ORFs. ORF1 spans approximately 87.5% of the entire genome, whereas ORF2 is small. Overlap exists between ORFs. B) Predicted protein structure of the walrus calicivirus CV-HMU-1. ORF1 contains eight conserved calicivirus amino acid motifs and four conserved protein domains, according to the Conserved Domain Database of NCBI. (S1 Fig shows the domain architecture predicted by NCBI CD-Search for CV-HMU-1's full-length nucleotide sequence.) C) Secondary structure model for the 5'-UTR of CV-HMU-1. The structure was modeled with the aligned RNAalifold server of ViennaRNA Web Services. D) Secondary structure model for the 3'-UTR of CV-HMU-1. NCBI, National Center for Biotechnology Information; ORF, open reading frame; UTR, untranslated region.

CV-HMU-1 is a new member of the *Salovirus* genus of the *Caliciviridae* family (Fig 2). However, different evolutionary relationships were observed between *Salovirus*

and other genera, on the basis of VP1 and VP2. VP1 amino acid sequence analysis revealed that *Salovirus* and *Minovirus* share a closer phylogenetic relationship

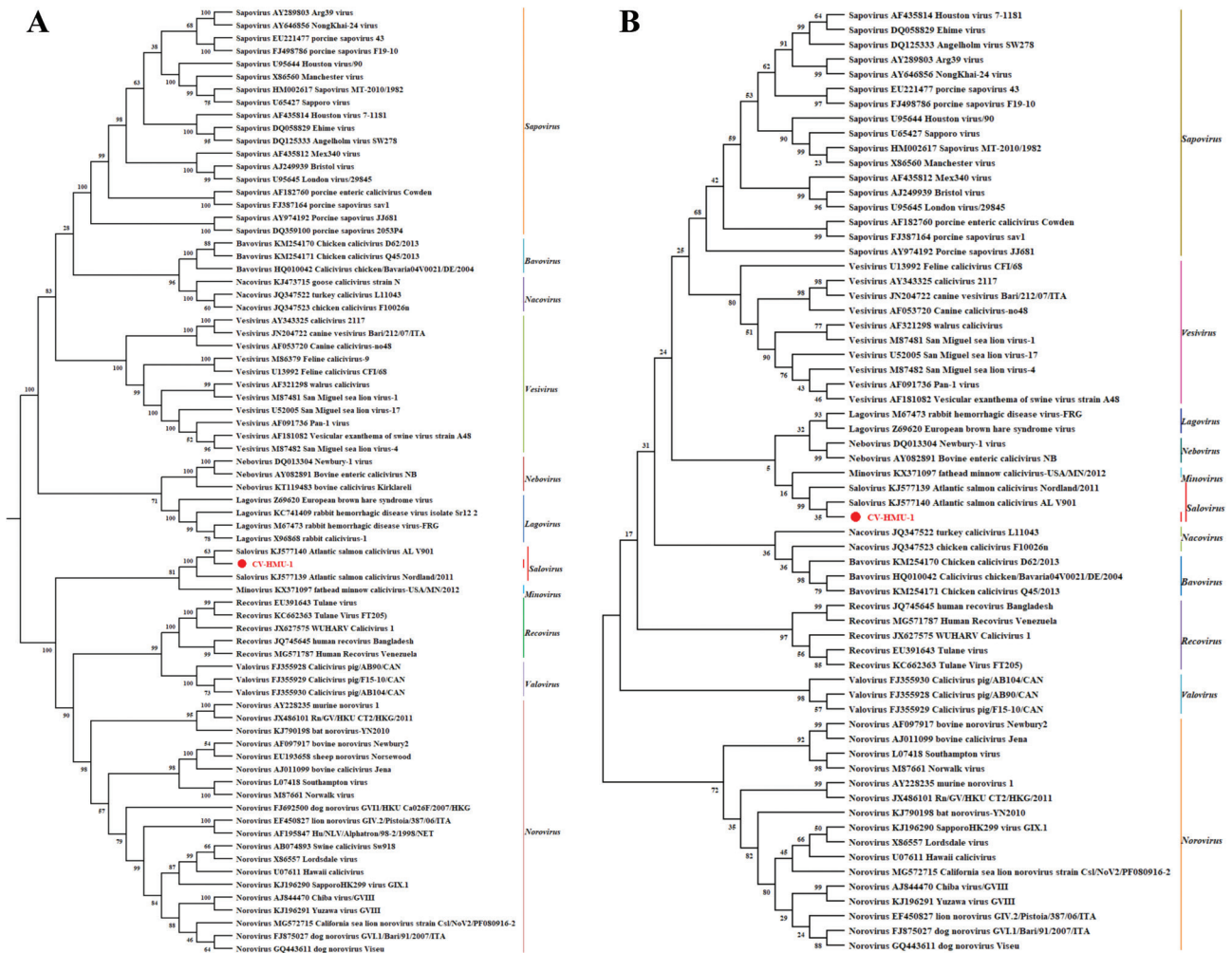


FIGURE 2 | Phylogenetic analysis of CV-HMU-1 and *Caliciviridae* family members.

A) Phylogenetic analysis based on the partial VP1 amino acid sequences of 74 caliciviruses. B) Phylogenetic analysis based on the VP2 amino acid sequences of 64 calicivirus strains.

with each other (Fig 2A, S2 Fig) than either does with *Nacovirus*; this contrasts with VP2-based clustering, where *Salovirus*/*Minovirus* exhibit greater proximity to *Nacovirus* (Fig 2B, S3 Fig).

DISCUSSION

Caliciviruses are important zoonotic pathogens that infect many species and have a wide distribution. Calicivirus transmission within the same or different host species is influenced by overlapping living environments, and is enabled by direct contact with infected hosts or indirect contact with fecal material, vomitus, respiratory secretions, contaminated food, water, or fomites. HuNoV genogroup II, porcine SaV, and bovine NoV have been detected in shellfish samples from 45 bays in the United States; in that study, five sequenced HuNoV GII-positive oyster tissues were similar to the NoV GII US 95/96 subset (GII-4) in the polymerase region, which has previously been implicated in diarrhea outbreaks [31]. These findings have suggested that HuNoV outbreaks are associated with shellfish

consumption. In addition, the bat calicivirus BtCalV/A10/USA/2009 identified in 2018 is similar to HuNoV in its capsid sequences, and its receptor-binding domains have retained host ligand binding. The pattern of the two calicivirus-binding tissue blood group antigens overlapped with those of human and animal NoVs. Therefore, bat caliciviruses can bind tissue blood group antigens and overcome important barriers for cross-species transmission [32]. *Vesivirus* is the most common calicivirus exhibiting spread across species; for example, approximately half of the marine vesiviruses have been documented to produce skin vesicles in swine and horses [33].

In this study, we screened 18 specimens from a marine mammal sample pool that had tested positive for *Caliciviridae*-related sequences in preliminary assays and found only one calicivirus-positive anal swab (5.56%). Our findings might indicate that this calicivirus infection is transient or asymptomatic. Calicivirus transmission occurs via the fecal-oral route, and the pathogen can be foodborne or waterborne. However, the oral and nasal swab samples from the walrus with the positive anal

swab yielded negative findings, possibly because they did not contain sufficient virus-infected shed host cells for detection. Whether the newly discovered calicivirus CV-HMU-1 might be the direct cause of diarrhea in walrus requires further investigation.

Herein, we collected a total of 109 swab samples from marine mammals raised in Qingdao Polar Haichang Ocean Park and Dalian Sunasia Tourism Holding Co., Ltd., China (S1 Table), including belugas (*Delphinapterus leucas*) [24], walruses (*Odobenus rosmarus*), California sea lions (*Z. californianus*) [23], American sea lions, and bottlenose dolphins [34]. Each species-specific pool was processed with identical nucleic acid extraction protocols and viral metagenomic sequencing pipelines. Notably, calicivirus sequences were detected exclusively in the walrus sample pool. Among 18 samples tested from walruses (*Odobenus rosmarus*) in this study, only one positive result was obtained from a sample pool containing swabs collected at different time points from two different walruses. Therefore, the 5.6% positivity rate was observed strictly among the 18 walrus swab samples (n=18) in this study. In the entire cohort of 109 marine mammal swab samples (n=109), the CV-HMU-1 positivity rate was calculated to be 0.9% (1/109).

On the basis of our phylogenetic results, CV-HMU-1 is the first member of the genus *Salovirus* identified in marine mammals. Other marine members include ASCV from Atlantic salmon [27] and *Ameiurus nebulosus calicivirus 1* from the brown bullhead, *Ameiurus nebulosus* [30], which showed 77.39% and 65.63% amino acid sequence identity, respectively, with respect to CV-HMU-1 ORF1, according to BlastP. In phylogenetic analysis, CV-HMU-1 and fish calicivirus formed a relatively independent cluster, thus indicating that this novel virus might have originated from the food of walruses. Our analysis revealed significantly higher amino acid sequence homology between fish caliciviruses (ASCV and *Ameiurus nebulosus calicivirus 1*) than between these fish caliciviruses and CV-HMU-1. CV-HMU-1 might potentially be a walrus calicivirus; therefore, further screening and identification of more samples are needed.

The genomic structure of CV-HMU-1 was comparable to that of ASCV. Both viruses were found to contain two ORFs, ORF1 and ORF2, with a small overlapping fragment between them (175 nt in CV-HMU-1 and 21 nt in ASCV). The important calicivirus NS protein amino acid motifs of CV-HMU-1 were also similar to those of ASCV, and included two NTPase/helicase motifs (GLPGVGKT and GPPGCGKT), a VPg motif (DEYEL), a 3C-like protease motif (GDCGLP), and three RdRp motifs (YGDD, GLPSG, and DFGRWDST); however, the motif positions were found to differ between these viruses. Individual amino acid differences were observed in the motifs among various calicivirus genera (S2 Table). Overall, our findings suggested that CV-HMU-1 is a novel species of *Salovirus*.

With improvements in detection technology, novel caliciviruses have been identified in birds, fish, cats, and many other vertebrates. *Caliciviridae*, as RNA viruses in

vertebrates, tend to broadly follow the evolutionary history of their hosts, beginning in the ocean and extending for hundreds of millions of years. Fish viruses branched earlier than those from other vertebrate groups (amphibians to mammals), reflecting their divergent phylogenetic position [35]. Therefore, we hypothesize that CV-HMU-1 may have evolved from ASCV or *Ameiurus nebulosus calicivirus 1* via host-predator transmission, given that CV-HMU-1 exhibits significantly higher genetic similarity to ASCV than to other known caliciviruses. In summary, the identification of this novel calicivirus should provide important insights for understanding calicivirus genetic diversity and preventing viral diseases.

DATA AVAILABILITY STATEMENT

The raw sequence data have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive under accession number PRJNA650224. The complete nucleotide sequence data of CV-HMU-1 have been submitted to NCBI GenBank, under accession number OQ110727.

ETHICS STATEMENT

The sampling procedures were approved by the Ethics Committee of Hainan Medical University (approval number: HMUEC20180059). The animals were treated according to the guidelines of the Regulations for the Administration of Laboratory Animals (Decree No. 2 of the State Science and Technology Commission of the People's Republic of China, 1988).

AUTHOR CONTRIBUTIONS

Yi Huang, Peijun Zhang, and Feifei Yin designed this study. Yi Huang, Zihan Li, Dan Ren, Gaoyu Wang, Shijie Bai, Yun Zhang, Ruoyan Peng, Xiaoyuan Hu, Youyou Li, Xin Li, Chuanning Tang, Gang Lu, and Peijun Zhang collected specimens and performed the experiments. Yi Huang, Gaoyu Wang, Dan Ren, Zihan Li and Feifei Yin analyzed the data. Yi Huang, Yun Zhang, Zihan Li, Dan Ren, and Gaoyu Wang wrote the original draft. Peijun Zhang, Feifei Yin, and Yun Zhang revised the manuscript. All authors have read and approved the final manuscript.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

SUPPLEMENTARY MATERIAL

Supplementary Material can be downloaded from https://zoones-journal.org/wp-content/uploads/2025/07/zoones20250002_Suppl.zip.

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