Detection of vesivirus in minks (Neovison vison)*, Italy 2021*

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Summary
Vesiviruses are important animal pathogens with a broad host range, and they have also been involved in accidental contamination of cells used for the production of drugs for rare and life-threatening human diseases. A vesivirus (family *Caliciviridae*) was detected in minks (*Neovison vison*) with respiratory and neurological signs, during syndromic surveillance for SARS-CoV-2 conducted in Italy. The complete genome (8,397 nucleotides in length) of the vesivirus strain ITA/2021/mink/TE (OR130287) was obtained by combining NGS approach with 5′ and 3′ RACE protocols. The virus was seemingly more related (95.9-97.2% nt identity in the partial RNA-dependent RNA polymerase) to American vesivirus isolates 9/1980/US, 12/1980/US, and 20/1980/US dating back to the early 1980s than to recent mink strains. These results highlight the importance of gathering information on the virome of animals.

The emergence of new human pathogens is nowadays regarded as a global threat to human health and the trajectory of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a paradigm of the one health concept. SARS-CoV-2 is regarded as a pan-zoonotic virus, able to infect companion animals, captive exotic animals in zoos, sanctuaries, and aquariums and free-ranging wildlife (Ghai *et al.*, 2021; World Organization for animal health, 2019; Mabry, M. E., Fanelli, A., Mavian, C., Lorusso, A., Manes, C., Soltis, P. S., & Capua, I. (2023). The panzootic potential of SARS-CoV-2. Bioscience, 73(11), 814–829. https://doi.org/10.1093/biosci/biad102). The ability of SARS-CoV-2 to infect mink (*Neovison vison*) has impacted heavily on the fur industry globally. Between 2020 and 2021, after the first SARS-CoV-2 outbreak in a Dutch mink farm in April 2020 (Oreshkova *et al.*, 2020), SARS-CoV-2 infection has been described in 478 mink farms from 13 countries (World Organization for animal health, 2019). Animal practitioners of mink farms were regarded as the major source of infection for minks. Also, spill-back, mink-to-human transmission, cases of infection have been reported in Europe (Oude Munnink *et al.*, 2021; Hammer *et al.*, 2021; Larsen *et al.*, 2021; Rabalski *et al.*, 2022) and Canada (Paiero *et al.*, 2022) with viruses of mink origin raising concerns for the emergence of new SARS-CoV-2 variants resistant to vaccines and antivirals or with increased virulence in the human population (Tan *et al.*, 2022). The risk of infection of SARS-CoV-2 in minks has solicited the health authorities to enforce stricter prophylaxis measures, including higher biosecurity and structured surveillance activities.

Surveillance for SARS-CoV-2 in minks in Italy has been enacted promptly in the spring of 2020 and intensified in 2021, combining passive and active measures. Passive (syndromic) surveillance was based on voluntary notification of disease in herds, whilst active surveillance was based on structured monitoring of mink herds as outlined in EU recommendations (EU Decision 2021/788 of 12 May

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2021). Taking advantage of the screening for SARS-CoV-2, we implemented the diagnostic algorithms with a metagenomic pipeline that was applied on a selection of samples collected in the mink farm, to gather more information on the virome of these animals.

One mink herd, located in Castel di Sangro, Abruzzi region, Central Italy (with about 230 males and 830 females), was monitored since mid-2020 by the Istituto Zooprofilattico of Abruzzi and Molise (IZS AM). Overall, a total of 720 (n=60 per month) samples (oral swabs and carcasses) were tested in 2021 using a specific quantitative assay for SARS-CoV-2 RNA (TaqManTM 2019-nCoV Assay Kit, Thermofisher), but SARS-CoV-2 was never detected in the surveyed animals.

In June 2021, respiratory distress and neurological (lethargy) signs were reported in the mink herd, with notification to the health authorities, but the samples (oral swabs) tested negative for SARS-CoV-2 and the case was dismissed. A sample from this case was subsequently included in a retrospective metagenomic investigation that included a selection of samples collected during surveillance activities. Total RNA was extracted using QI-Aamp Viral RNA Kit (Qiagen, Hilden, Germany) and PCR-enriched using a sequence-independent (SISPA) protocol (Marcacci *et al.*, 2016). The libraries were constructed using Illumina® DNA Prep (M) Tagmentation (96 Samples) (Illumina Inc., San Diego, CA, USA), according to the manufacturer's protocol. Deep sequencing was performed on the NextSeq500 (Illumina Inc., San Diego, CA, USA) using the NextSeq 500/550 Mid Output Reagent Cartridge v2 (300 cycle) (Illumina Inc., San Diego, CA, USA) and standard 150 bp paired-end reads. After a quality check and trimming of raw reads data using FastQC v0.11.5 and Trimmomatic v0.36, respectively, host depletion was performed by Bowtie2 (Lorusso *et al.*, 2020). The sequence (fastq) files generated were uploaded on CZ-ID (https://czid.org/), which results in the assignment of reads and contigs to taxonomic categories.

On metagenomic analysis, the sample yielded n=235,213 reads and most (n=178,028, 75.7%) were classified as *Mycoplasma* spp. (M. *mustelae*, M. *felis*, M. *gallinaceum*, M. *NEAQ87857*, M. *edwardii*, M. sp) whilst n=15,533 reads (6.6%) were classified as *Vesivirus*. The genome of the vesivirus (VeV) strain (ITA/2021/mink/TE) was assembled using Geneious Prime (Dotmatics, New Zealand). The reads were mapped to a reference sequence using MiniMap2 (Li, 2018) and the gaps were filled with an overlapping strategy whilst the genome terminations were sequenced using 5' and 3' RACE protocols (Scotto–Lavino *et al.*, 2006). The genome sequence was deposited in GenBank under accession nr OR130287. The genome of strain ITA/2021/mink/TE was 8,397 nt long and displayed 92.5% nt and 90.5% nt identity to the Chinese VeV China/2/2016 (accession MF677852) and DL/2007/CN (accession JX847605), respectively. Also, the virus displayed 95.9-97.2% nt identity to partial (464 nt-long) sequences of the RNA-dependent RNA polymerase (RdRp) of VeV strains 9/1980/US, 12/1980/US, and 20/1980/US, detected in minks in USA (accessions AF338406, AF338407 and AF338408, respectively). Genome identity to other VeVs was lower than 70% nt. Phylogenetic analyses confirmed the close relatedness of strain ITA/2021/mink/TE to other VeVs detected from minks and badgers, although clustering within a distinct clade (Figure 1).

VeVs are small non-enveloped RNA viruses classified in the genus *Vesivirus*, family *Caliciviridae*, identified in several animal species, including aquatic and terrestrial mammals, and reptiles (Vinjé *et al.*, 2019). Prototypes of this genus are feline calicivirus (FCV), San Miguel Lion virus (SMLV), and vescicular exantema swine virus (VESV) (Vinjé *et al.*, 2019). Based on sequence and phylogenetic analyses of the full-length VP1 capsid gene (ORF2), VeVs are genetically heterogenous and segregate into different genetic groups (Martella *et al.*, 2015). Recent investigations in wildlife have discovered VeVs in other mustelids, including ferret badger (*Melogale moschata*) (Miao *et al.*, 2015), European badger (*Meles meles*) (Reuter *et al.*, 2023) and Asian badger (*Meles leucurus*) (He *et al.*, 2022), thus enlarging the known host range of these viruses.

In minks, VeVs were first isolated from normal animals on ranches with a history of hemorrhagic pneumonia in 1980 (Long *et al.*, 1980). However, only 20 years after their initial discovery, the isolates 9/1980/USA, 13/1980/USA, and 30/1980/USA were finally characterized as members of the *Vesivirus* genus, based on partial sequences of the RdRp, and were found to differ from uncultivatable mink enteric caliciviruses (Guo *et al.*, 2001). The VeV strains were grown in Vero cells and were antigenically distinct from other VeVs, i.e. VESV, SMSV, and FCV by neutralization tests (Long *et al.*, 1980). Also, infection by mink VeV appeared widespread in mink population, as neutralizing antibodies were detected at a prevalence of 80-100% in commercial and institutional herds from USA and Japan (Long *et al.*, 1980). The complete genome sequence of a mink VeV, strain DL, was determined only in 2012 from a disease outbreak in mink in 2007 in Shenyang Province, northeastern China (Yang *et al.*, 2012). An additional strain, MCV-GCCDC8-2020, was isolated and sequenced from Hebei Province, China, 2020, on testing of anal swabs of deceased minks (Guo *et al.*, 2021). Also, the partial genome sequence of a VeV strain was obtained serendipitously on metagenomic sequencing of mink samples during a study on SARS-CoV-2 in France (Wasniewski *et al.*, 2023).

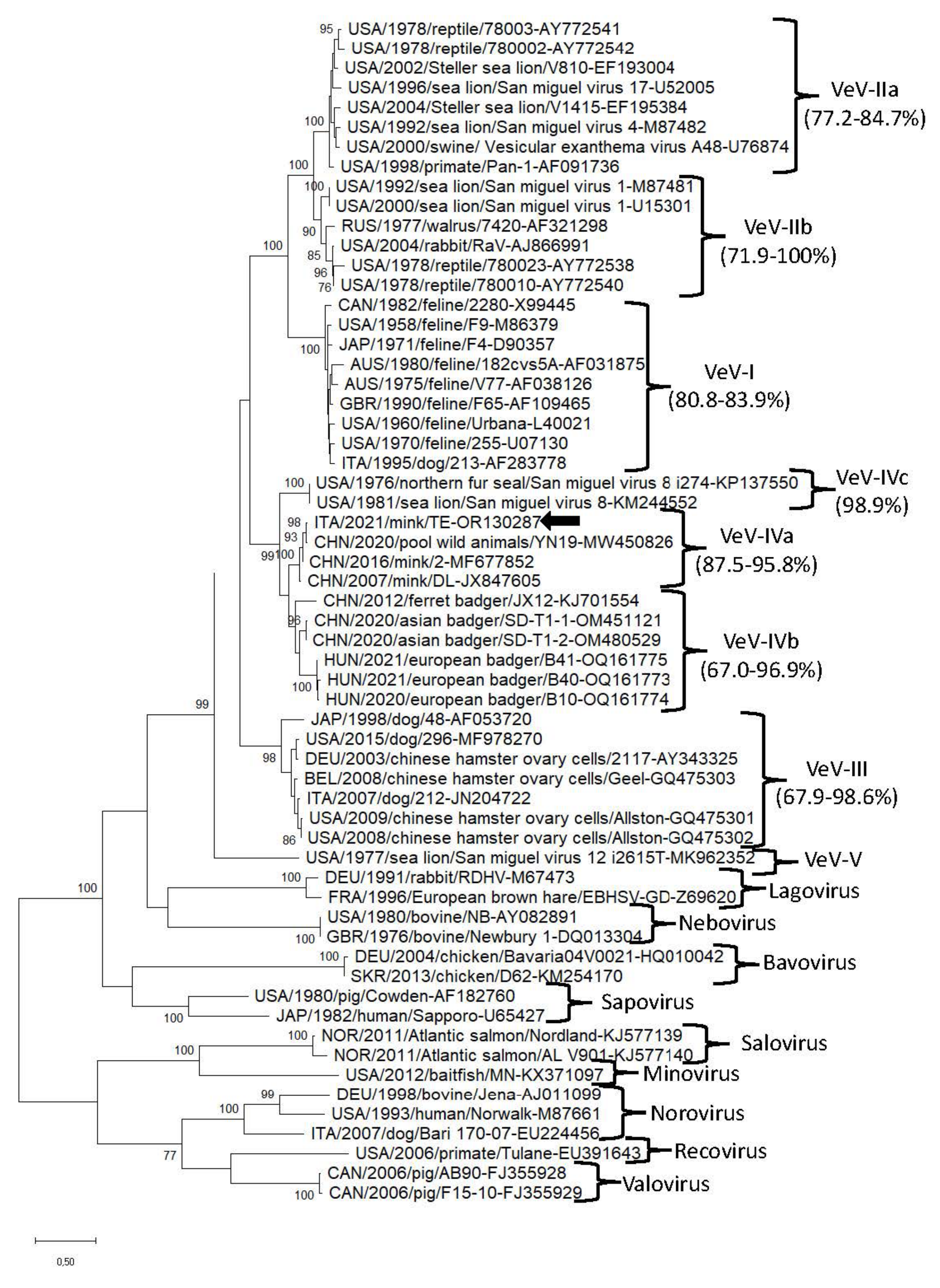

Figure 1. *Phylogenetic tree based on the complete amino acid sequence of the capsid protein of vesivirus (VeV) strain ITA/2021/mink/TE identified in this study along with cognate sequences of established and candidate calicivirus genera available in the GenBank database. The Maximum Likelihood method and LG+G+F model based on 327 amino acid positions were used for the phylogeny. One thousand bootstrap replicates were used to estimate the robustness of the individual nodes on the phylogenetic tree. Bootstrap values higher than 75% are displayed. Black arrow indicates the mink VeV strain ITA/2021/mink/TE. VeV groups were defined on the basis of distance matrix comparison and phylogenetic clustering. The mean identity among strains of the main genetic groups (indicated by Roman numerals and a letter or both) is shown. The scale bar represents the number of amino acid substitutions per site.*

All in all, these findings indicate the VeV infection in these animals is not uncommon and reveal a substantial genetic conservation, hinting to a species-specific pattern/restriction (Figure 1).

Although initially associated with hemorrhagic pneumonia of mink, observational findings and experimental data, thus far, have not been conclusive to decipher the pathogenic role, if any, of VeVs in minks. Experimental infection of ten mink kits (10 to 12 weeks old) with a mink VeV isolate did not reproduce clinical signs or lesions (Long *et al.*, 1980). Likewise, experimental infection of minks with SMSV did not induce clinical signs. Except for one mink with vesiculation at the site of intradermal inoculation, the infection was inapparent. However, minks had no detectable virus-neutralizing antibody to SMSV before infection and developed titers of 1:4 to 1:16 after infection, suggesting active immunization (Wilder and Dardiri, 1978).

In 2003, 2008 and 2009, episodes of contamination by VeVs (strain 2117-like) of cell cultures used for the production of recombinant drugs have been reported but the origin of contamination remained unknown, although similar VeVs have been subsequently identified in dogs (Martella *et al.*, 2015). Antibodies specific for canine VeV have been detected using an ELISA assay in 32/410 (7.8%) human sera, indicating human exposure to VeVs (Di Martino *et al.*, 2015). However, due to the possibility of cross-antigenic reaction in ELISA between different VeV strains/clades, the exact source of exposure for the human population remains uncertain. Gathering information on the virome of animals is pivotal to understanding better the ecology of VeVs and assessing the zoonotic risks.

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