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Emerging Protoparvovirus in Pigs and Wildlife, Denmark

Appendix

Appendix Methods

Microorganisms monitored in the investigated pig herd. The herd is annually tested for *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae* 2, 6, and 12, porcine reproductive respiratory syndrome virus 1 and 2, *Brachyspira hyodysenteriae*, *Pasteurella multocida*, *Sarcoptes scabiei* var. *suis*, and *Haematopinus suis*.

Treatment of affected weaners. As the piglets showed unspecific clinical signs, they were treated against both enteric and respiratory disease: doxycycline, an acid neutralizer, and an anti-inflammatory were dispensed in the water, and piglets were individually treated with sulfadoxin, trimethoprim, or florfenicol.

Wildlife sampling. This investigation included samples previously harvested from wild carnivorans, hunted or road-killed throughout Denmark, within the context of the annual Danish national passive wildlife disease surveillance program, performing disease surveillance throughout the country. Post-mortem examinations were performed by experienced veterinary pathologists to assess overall external and internal body conditions. Tissue sampling was performed during routine necropsy procedures, and the samples were collected only from animals in adequate post-mortem condition (i.e., not showing signs of advanced decomposition). During sampling, sterile, single-use instruments and containers were employed to avoid cross-contamination, and all tissue samples were stored at −80°C in a dedicated tissue archive until further analysis.

Tested samples included 2 fecal and 80 spleen samples from red foxes (*Vulpes vulpes*), 3 spleens from raccoon dogs (*Nyctereutes procyonoides*), 10 spleens from Eurasian otters (*Lutra*

lutra), 6 spleens from beech martens (*Martes foina*), 5 spleens from European badgers (*Meles meles*), 2 spleens from pine martens (*Martes martes*), and 1 spleen from a polecat (*Mustela putorius*).

Virus detection and genome sequencing. DNA was isolated from tissue samples (~10mg of spleen or ~20mg of liver and kidney) using the Qiagen DNA blood and tissue kit according to the manufacturer's specifications. Isolated DNA was used to perform a semi-nested PCR using primers designed in this study to anneal to all so far identified viruses within the species *Protoparvovirus carnivoran4*: Fox_Parvo_F1: ACTGTGGTTCGCATAGGCTG, Fox_Parvo_R4: CTTGGATAGTAGACCAGGTTG; Fox_Parvo_R1: GTTCAGCAGTTGGCTTGG-TG). PCRs were performed using the DreamTaq PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) in 25µl volumes using 0.5 ul of 10 µM primers and 2.5 µl cDNA as input. Amplifications were run at 95°C for 5 minutes, followed by 35 (first step) or 25 (nested) amplification cycles performed for 30 seconds at 95°C, 30 seconds at 50°C, and 30 seconds at 72°C, followed by a final amplification step at 72°C for 7 minutes. The nested PCR was only performed if the first PCR was negative, as visualized on a 1% agarose gel.

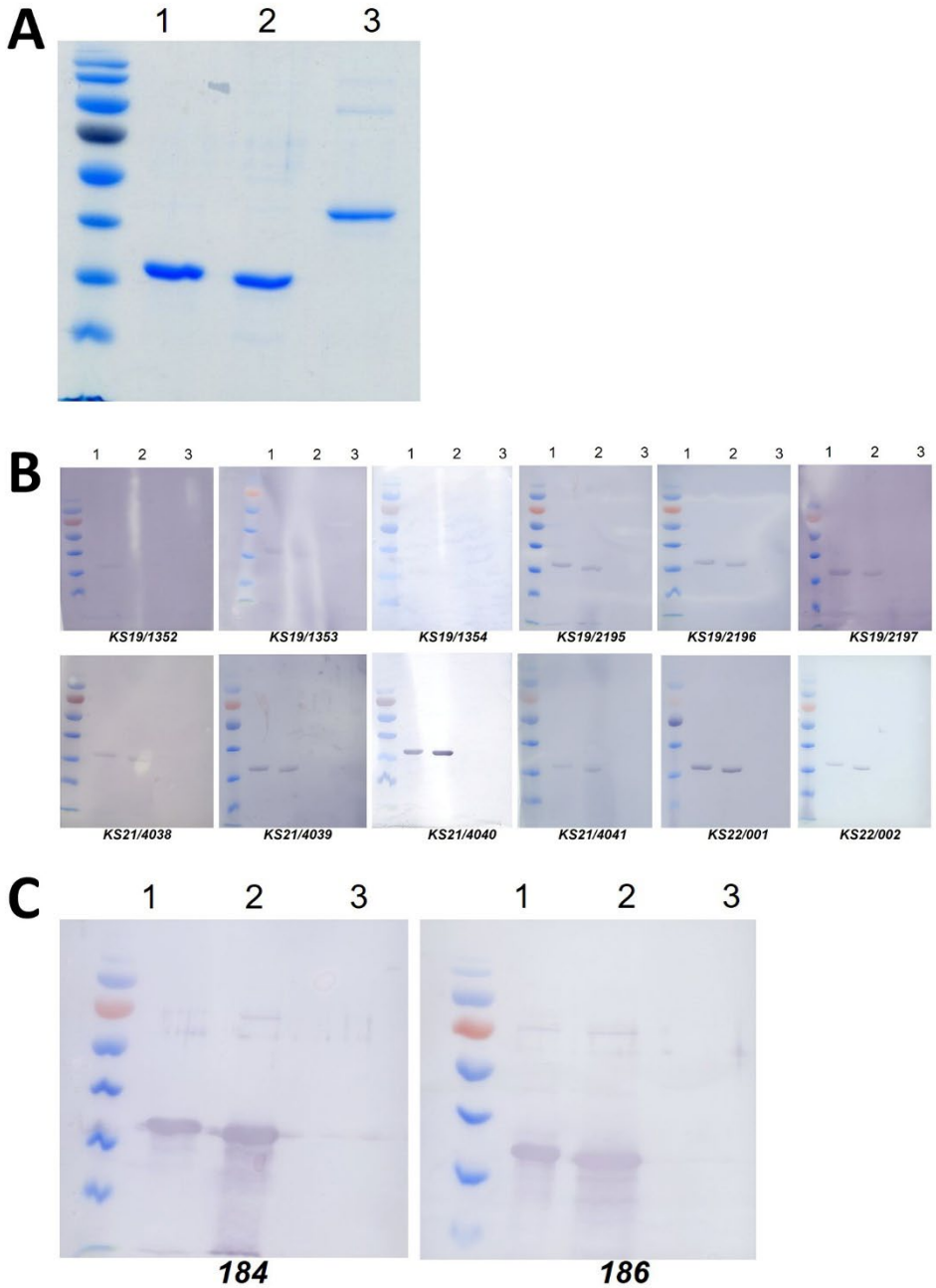
Four overlapping PCRs were designed to obtain the full genome sequences of the identified viruses (Appendix Table).

Obtained amplicons were purified with the HighPrep PCR-DX purification beads (MAGBIO Genomics) and outsourced for Sanger sequencing.

Sequence analyses: Low-quality sequences and primer binding regions were removed from the reads, and these were assembled to obtain the full sequence. Multiple sequence alignments between study sequences and reference sequences downloaded from the GenBank database were created using MAFFT and used for building a maximum-likelihood phylogenetic trees with IQ-TREE3 using the best-fitting model for genetic distance estimation. Branch robustness was assessed through the Shimodaira–Hasegawa approximate likelihood-ratio test and ultrafast bootstrapping (1000 replicates). Full-genome alignments were also used to make similarity plots (Window: 200, Step: 20) across the genome and full-genome pairwise sequence identity matrices. All these analyses were performed within the framework of Stranded (VerCan ApS).

Appendix Table. PCR primers used

No.	length	Forward primer	Reverse primer
1	1,232 bp	F3: AACTAAGCTTTACCATGTAAGG	R3: ATTGGATTCCATCCATGTCCC
2	1,057 bp	F4: GACTGGATGATGAATGCACCA	R4: CTTGGATAGTAGACCAGGTTG
3	1,845 bp	F1 ACTGTGGTTCGCATAGGCTG	R5: GGCTTGCAGTGAGGTCATTG
4	1,543 bp	F6: ACCAGATGGTTACTCCGTGG	R6: GATATAGCATATAGAAACCTCCC

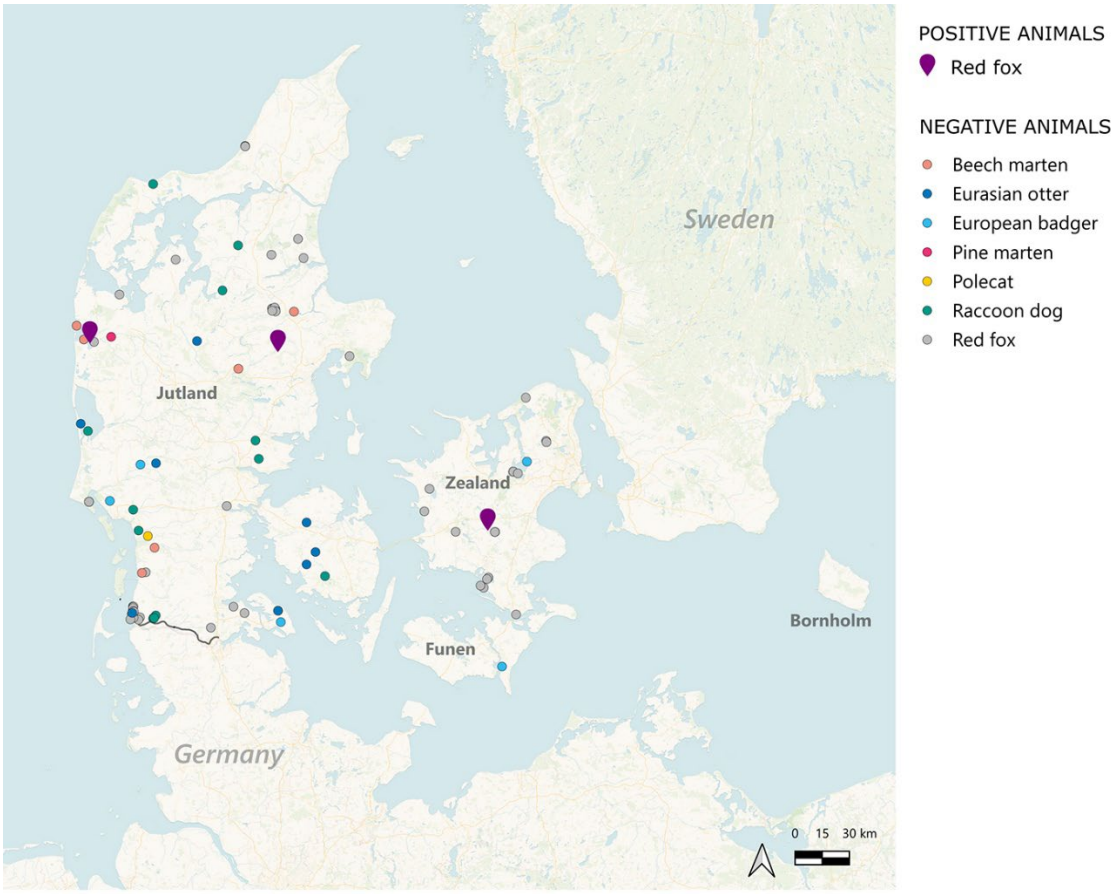


Appendix Figure 1. Phylogenetic analysis of members of the genus *Protoparvovirus*. Aligned non-structural (NS1) protein sequences were used to build the maximum-likelihood tree with IQTree3 according to the Q.YEAST+F+I+G4 model, identified as the best fitting for genetic distance estimation

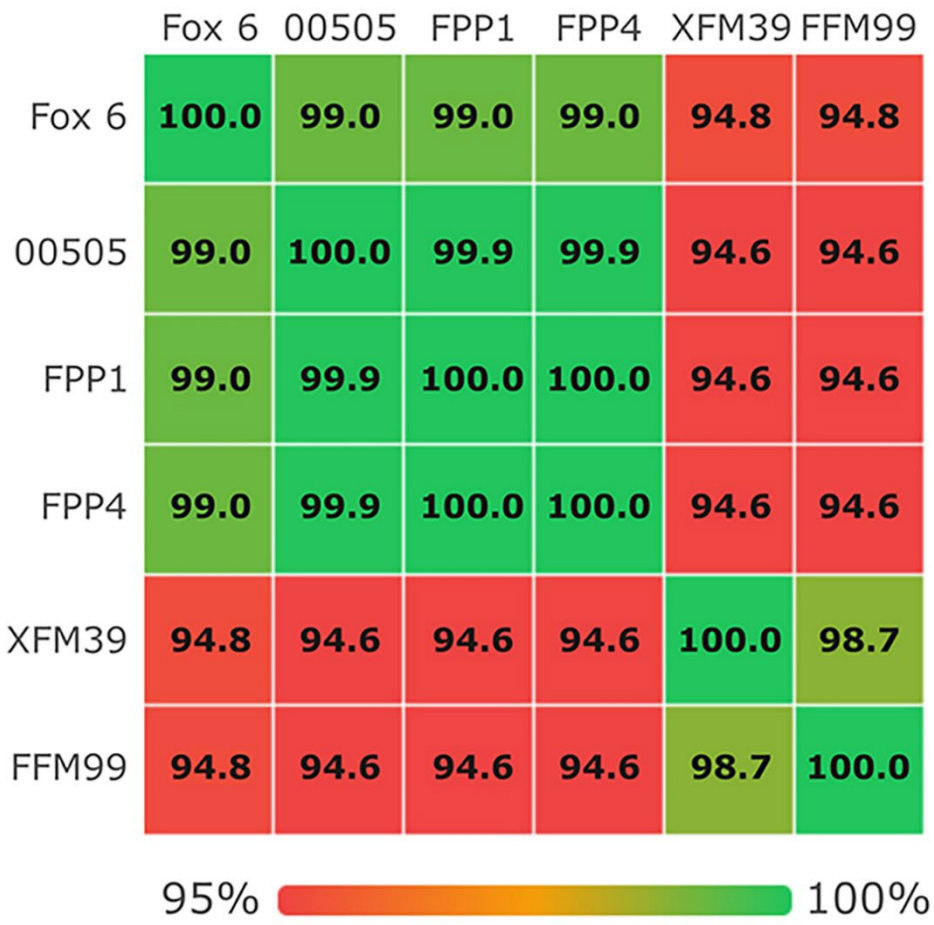
(modelfinder function). The outcomes of the Shimodaira–Hasegawa approximate likelihood-ratio test and ultrafast bootstrapping (1000 replicates) are indicated at the main nodes. Amdoparvovirus sequences were used as outgroup. For each species, indicated on the right, the ICTV designated prototypical virus was used. Members of *Protoparvovirus carnivoran4* are labeled in red. The analysis was performed with Stranded (VerCan ApS).



Appendix Figure 2. Typical clinical signs in pigs affected by fox parvovirus. Infected piglet showing signs of exophthalmos, eyelid edema, and strabismus.



Appendix Figure 3. Sample collection locations across Denmark. Locations are indicated by colored circles corresponding to different animal species, as indicated in the legend, and positive animals are indicated by pins.



Appendix Figure 4. Pairwise nucleotide identities among complete fox parvovirus genome sequences from pigs and foxes. Values indicate percentage nucleotide identity between sequences. Cell colors indicate sequence identity according to the scale, ranging from red for lower identity to green for higher identity. The analysis was performed with Stranded (https://www.vercan.dev).