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Virome analysis reveals ORF7 sequences of type 2 porcine respiratory and reproductive syndrome virus (PRRSV) for the first time in a rodent host (*Microtus pennsylvanicus*)

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ABSTRACT

The lung virome of meadow vole (*Microtus pennsylvanicus)* from six neighbouring locations in New York State, USA was investigated using metagenomics to determine the circulation of potentially zoonotic viruses in a common wild rodent. This study provides the first evidence of the occurrence of Type 2 Porcine Respiratory and Reproductive Syndrome Virus (PRRSV2) in a rodent, or indeed in any species apart from wild and domestic pigs (*Sus scrofa*). PRRSV has the highest economic impact on the pig husbandry industry of any pathogen, but up to now, farm-to-farm transmission of this virus has been assumed to be associated with movement of and contact with infected pigs and fomites. Our results showing the natural occurrence of this virus in potential transmission hosts other than wild or domestic pigs challenge this scenario. Phylogenetic analysis of assembled partial genomes from four of our pooled samples and all other nucleocapsid protein (ORF7) sequences available in Genbank showed that the sequences recovered from meadow voles unambiguously clustered within the PRRSV2 clade together with sequences derived from wild and domestic pigs. Historical research suggests that spillover from voles to domestic pigs may be the most parsimonious explanation for these results; however, we cannot rule out the reverse: that the source of PRRSV2 in these wild voles derives from pigs. From a One Health perspective, our results reinforce the importance of characterizing wildlife viromes to survey possible sources of zoonotic pathogens, which is vital for making evidence-based decisions regarding potential threats to the health of humans, livestock and wild fauna.

1. Introduction

Rodents are well-recognized reservoirs of zoonotic pathogens of public health relevance, including pathogens that have affected the pig industry [1,2]. However, to support a One Health approach, and address the intersection of wildlife, livestock and human health more rigorously, the contribution of wild rodent populations to zoonotic outbreaks in pigs (*Sus scrofa*) and other livestock deserves more attention. One example of an abundant wild rodent is the meadow vole (*Microtus pennsylvanicus*) found widely across northern North America [3]. The meadow vole moves between natural and human-modified habitats [4] and is host to a range of zoonotic pathogens, such as *Borrelia burgdorferi* and Hantaviruses [1,5,6]. It has previously been suggested that this rodent species could be linked to farm-to-farm transmission of pathogens affecting livestock [2], but to our knowledge this hypothesis has not been tested.

One particularly important pathogen affecting pigs is the Porcine

Abbreviations: PRRSV, Porcine Reproductive and Respiratory Syndrome Virus; PBS, Phosphate-buffered saline; EDTA, Ethylenediaminetetraacetic acid.

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Respiratory and Reproductive Virus (PRRSV), a single-stranded, positive-sense RNA-enveloped virus belonging to the *Betaarterivirius* genus of the family Arteroviridea [5,7]. The PRRSV genome consists of 15 kilobases, is polyadenylated at the 3′ end, and includes 11 open reading frames (ORFs) encoding seven proteins [7–9]. Clinical signs typical of infection are reproductive failure in pregnant sows and respiratory disease, ranging from mild to severe, in both newborn and juvenile individuals [8]. PRRSV has the greatest global economic impact of any pathogen affecting the pig husbandry industry, with fatality rates of up to 20%, and hundreds of thousands of deaths in single epidemic events, costing more than 0.5 billion USD per year in North America alone [10–13].

The first report of PRRSV signs in domestic pigs [14] indicates the pathogen was already present in Eastern and Mid-Western USA (North Carolina, Minnesota, Iowa and Indiana) [14] in the late 1980s, consistent with subsequent phylogenetic findings (see below). Currently, PRRSV is classified as having two genotypes, Type 1 (‘European’) and Type 2 (‘North American’), according to the sequence identity of the non-glycosylated membrane protein (ORF6) and the nucleocapsid protein (ORF7), both found only in wild and domestic pigs (*S. scrofa*) [8,15,16]. Phylogenetic analysis suggests these two Types emerged simultaneously in the late 1980s and early 1990s, in Europe and North America, respectively [8,15], but are now both known to be distributed globally. Despite similar signs, they share low nucleotide identity (50–70%) and their taxonomy places them on two distant branches of the Betaarterivirius genus, indicating that PRRSV is paraphyletic with a distant common ancestor [8,15,17]. Intriguingly, some of the most similar sequences to those of PRRSV in public databases such as Genbank are found in voles; however, to our knowledge, the connection between pigs and voles as hosts has not been established.

In the last decade, the development of viral shotgun metagenomics has allowed the characterisation of the entire community of microorganisms in a single biological sample (e.g. tissue or fecal sample), providing rapid detection and monitoring of pathogens in potential host species as well as the discovery of novel zoonotic strains [18,19]. Here, we investigate the virome of meadow vole lung tissue from six neighbouring populations in New York State, USA, to provide insight into the presence of zoonotic viruses in this species. Among these, we report the first evidence of PRRSV Type 2 (PRRSV2) in a species other than *S. scrofa*. Importantly, phylogenetic analysis suggests a potential spillover event between these taxa.

2. Materials and methods

2.1. Sampling

M. pennsylvanicus was collected from six locations north of Ithaca in Tompkins County, New York State (NW, SE, SW, Lo1–3; Fig. 1), 60 m to 3.3 km apart, using Longworth live-traps [20] between July 2015 and September 2016. Voles were euthanised on site using isoflurane followed by cervical dislocation under licence Protocol #2014–0046 (approved by the Institutional Animal Care and Use Committee of

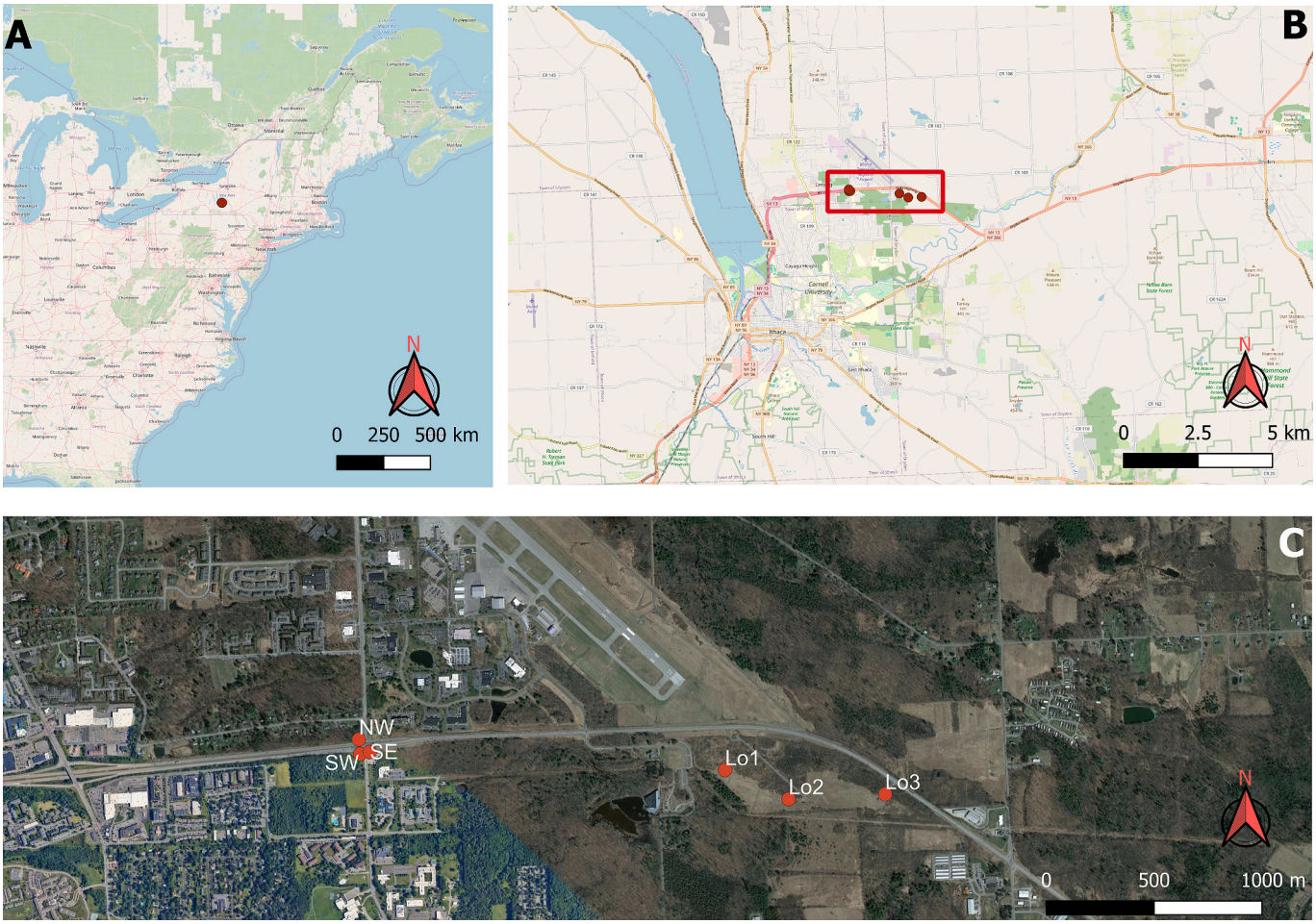

Fig. 1. *Microtus pennsylvanicus* sampling locations, indicated by red dots: A) map of eastern North America; B) map of sampling locations north of Ithaca, NY; C) satellite view showing distribution of sampling sites and nearby roads and urban/rural areas. Coordinates for the road intersection are: 42°28′55.9200″, −76°28′02.1000″. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Cornell University). Thoracotomy was followed immediately and the lungs, which showed no obvious signs of disease, were separated, placed in 1.5 ml Eppendorf tubes containing RNA Later (Thermo Scientific, Waltham, MA), and frozen at −20 °C for downstream processing.

2.2. Isolation of viral particles, RNA extraction and amplification

Laboratory analyses were performed at the Cornell Marine Mass Mortality Lab (CM3L). The lungs from all individuals trapped in each of the sampling locations were pooled in a sterile 50 ml Falcon tube containing virus-free (0.02 μm-filtered) PBS and sterile RNase-free lysis ceramic beads, for a total of six samples. The tubes were vortexed until tissue homogenization, centrifuged at 8000*g* for 5 min, and the supernatant filtered through a 0.2 μm Polytetrafluoroethylene syringe filter into a new sterile Falcon tube. This solution was incubated at 4 °C for 18–24 h after the addition of 10% by weight of Polyethylene glycol 8000. After incubation, the tubes were centrifuged at 8000*g* for 30 min, the supernatant discarded, and the pellet resuspended in 1 ml of virus-free (0.02 μm-filtered) PBS. To eliminate free-ranging co-extracted nucleic acids, the samples were incubated at 37 °C for 3 h with 2.5 U of Turbo DNase (Thermo Scientific, Waltham, MA), 0.25 U of RNAseOne (Promega, Madison, WI) and 5 U of benzonase nuclease (Sigma-Aldrich, St. Louis, MO). Subsequently, enzymatic activity was stopped by the addition of 0.2 volumes of 100 mM EDTA, for a final concentration of 20 mM EDTA. Samples were then stored at −80 °C until nucleic acid extraction.

The ZR Viral RNA kit (Zymo Research, Irvine, CA) was used to extract viral RNA in a final elution volume of 15 μl, following the manufacturer's instructions. Contaminant DNA was removed using the ZR-Duet DNA/RNA Mini Prep kit (Zymo Research, Irvine, CA), applying the 'In-column Dnase I Digestion' step. The Complete Whole Transcriptome Amplification (WTA2) Kit (Sigma-Aldrich, St. Louis, MO) was used to amplify the extracted viral RNA according to the manufacturer's instructions. The sequencing run was performed at the Cornell Biotechnology Resource Centre on an Illumina MiSeq platform with 2x250bp paired-end technology.

2.3. Bioinformatic data curation

Sequencing data were analysed using LAZYPIPE version 3.0 [18], an automatic bioinformatic pipeline. Quality control was initially performed on raw FASTQ reads using Trimmomatic V 0.40 [21] to remove low-quality reads and trim adapters with standard parameters. DeNovo assembly was performed with MEGAHIT v 1.2.9 [22]. A first round of annotations allowed the assemblies to be queried against the RefSeq.abv archaean, bacteria and virus database with Minimap2 [23]. The second annotation round employed BLASTN to map previously assigned viral assemblies against the RefSeq.vi, virus database and archaeans and bacteria assemblies against RefSeq.ab, bacteria and archaean database, and assigned NCBI taxonomy IDs. The contigs of PRRSV recovered by LAZYPIPE 3.0 were aligned against a full genome of PRRSV2 recovered from pigs in the Commonwealth of Virginia, USA (MN073127.1 GenBank) using Sequencher 5.4.6 (Gene Codes Corporation, Ann Arbor, MI, USA). Fine-scale phylogenetic analysis of PRRSV2 is usually performed by analysing the complete ORF5 sequence [10,24–26]; however, as it was not possible to recover complete ORF5 sequences in our samples, our analysis focused instead on the complete coding sequence of the nucleocapsid protein (ORF7). This was available in the majority of the samples, and was one of the sequences originally used to define PRRSV, as described above [24–26].

2.4. Phylogenetic analysis

A dataset was constructed by downloading all ORF7 PRRSV2 sequences from BLAST [27]. The dataset includes only sequences listing both associated host and sampling locations. From this list, we also removed duplicates, obtaining a final list of 1344 sequences, all from *S. scrofa*. In addition, two full ORF7 sequences from *Betaarterivirus sheoin* (KY369968.1), recovered from *Eothenomys inez* (Inez's vole) and *Betaarterivirus chinrav* (KP280006.1) recovered from *Myodes rufocanus* (grey red-backed vole) were added as an outgroup to root the tree, as they are considered the closest known relatives of the PRRSV2 [5]. The full dataset, including the sequences recovered here, was aligned using MAFFT [28] and standard parameters. The total number of sequences in the built dataset was 1350.

Phylogenetic analysis was conducted using IQTREE2 [29] and maximum likelihood inference on two datasets: the nucleotide and amino acid datasets, to verify whether the results remained consistent between alignments, as well as to identify any fixed substitutions in the *M. pennsylvaticus* sequences. For this analysis, we used 1000 ultrafast bootstrap replicates [30] and repeated the same analysis three times for both the nucleotide and amino acid datasets using different seeds.

Since too many short sequences can exacerbate homoplasy issues [31], to enhance the resolution of the backbone nodes and confirm the position of our samples within the phylogeny [32,33], a second nucleotide phylogenetic tree was built using a subsample of the original dataset [34]. This subset comprised 75 sequences: the two outgroup sequences *B. sheoin* (KY369968.1), and *B. chinrav* (KP280006.1) (File_S3.fas), the four sequences from *M. pennsylvanicus* generated here, and 69 sequences from the 1344 *S. scrofa* sequences downloaded from NCBI. The 69 sequences were chosen by arbitrarily selecting sequences evenly from across the phylogenetic tree (Supplementary Tree 1: selected sequences in red) to capture the full diversity of PRRSV2. The reduced phylogeny was then constructed from these selections using a slower but more accurate standard bootstrap.

Finally, to visually confirm the similarity of sequences, the distribution of the frequency of pairwise distances of the full ORF7 nucleotide alignment was plotted for: i) all PRRSV2 sequences isolated from *S. scrofa;* ii) the sequences isolated from *M. pennsylvanicus* vs. those from *S. scrofa*; iii) the outgroup sequences vs. all putative PRRSV2 sequences generated here and from *S. scrofa*. These p-distances were also plotted as a heatmap with hierarchical clustering in R. The p-distance calculated for specific genes is commonly employed to differentiate between viral serotypes or genotypes [26,35–37].

3. Results

3.1. Samples and PRRSV2 detection

Between 11 and 27 (mean = 21) *M. pennsylvanicus* individuals were collected from each of the six sampling locations (Table S1). Partial fragmented PRRSV2 genomes were recovered in all six samples (Table S1, Supplementary files on GitHub). In four out of six locations (NW, Lo1, Lo2, Lo3), a complete ORF7 usable for downstream analyses was recovered (assemblies deposited in the NCBI SRA under BioProject PRJNA1259483).

The presence of PRRSV2 genomes in the samples cannot be attributed to contamination deriving from human contact with pigs or pig farms. None of the field workers or laboratory researchers, nor any of their equipment or laboratory materials, came into contact with pigs or pig farms around the time of this study. There has never been any research on pigs in the laboratory where the work was carried out.

3.2. ORF7 genetic diversity and phylogenetic relationships

For the ORF7 gene, the nucleotide alignment for all 1348 putative PRRSV2 sequences included 380 base pairs, with 255 informative sites, 54 singletons, and 73 constant sites (File_S1.fas). The amino acid alignment (File_S2.fas) consisted of 126 sites, including 84 parsimony-informative sites, 28 singleton sites, and 14 constant sites. In the aligned amino acid sequences, five sites were identified for which alternative amino acids were fixed in either *S. scrofa* or *M. pennsylvanicus*

(Table S2).

Although the relatively short ORF7 fragment provided limited information for constructing a phylogenetic tree, it was adequate to determine whether our sequences could be classified as PRRSV2, and if they represented a previously undetected *Betaarterivirius* clade. The phylogeny constructed with the nucleotide alignment (Supplementary tree 1) was concordant with that of the amino acid alignment (Supplementary tree 2). In fact, the key nodes of the tree remained consistent across all three nucleotide alignment runs; minor differences were observed only in the placement of branches with low support, as expected. Although in all cases node support for the position of the *M. pennsylvanicus* clade within the *S. scrofa* phylogeny was low, probably due to the relatively low number of polymorphic sites, *M. pennsylvanicus* PRRSV2 clade forms a monophyletic group consistently nested among other well-supported *S. scrofa* PRRSV2 clades, creating a polytomy (Fig. 2). Therefore, our results suggest that this new clade should be included within PRRSV2. However, there is no clear geographic structuring evident in the PRRSV2 phylogenies (Supplementary trees 1 and 2).

Fig. 2B illustrates the three distributions of the frequency of p-distances. In viruses, the p-distances between genotypes/strains are expected to be higher than the highest p-distance calculated within a genotype/strain [38–41]. Instead, the distribution of p-distances within *S. scrofa* (blue bars; mean 0.0876 and median 0.0934) substantially overlaps with the distribution of p-distances calculated between the *M. pennsylvanicus* and *S. scrofa* sequences (orange bars; mean 0.1209 and median 0.1212). Additionally, when comparing these two (blue and orange) distributions in Fig. 2B to the frequency of p-distances calculated between the outgroup and all other putative PRRSV sequences (black bars), we observe a mean and median of 0.315 with no overlap (See Table S3 for further details). Therefore, this result also confirms the inclusion of the *M. pennsylvanicus* sequences within PRRSV2. Interestingly, the *S. scrofa* PRRSV2 distances (Fig. 2B: blue bars) include two peaks: the upper (lower) peak likely arose from a number of closely related sequences generated from samples collected during a local outbreak or survey, while the lower peak encompasses the majority of *S. scrofa* PRRSV2 diversity, with a mean of 0.088, a median of 0.093.

The heatmap derived from the distance matrix of the downloaded PRRSV sequences (Fig. S1) yielded results consistent with the phylogenetic analyses presented in Fig. 2A; that is, the *M. pennsylvanicus* PRRSV2 are closely related to each other (small blue area, lower right, Fig. S1), but are less distant from some *S. scrofa* sequences (yellow areas) than some *S. scrofa* are from each other (red areas lower right).

4. Discussion

Wild animal species often contribute to the introduction and maintenance of pathogens in agricultural systems, which may cause endemic infections in livestock and human populations [42]. However, even for economically important diseases in animal husbandry, the source is often unrecognized, and the contribution of surrounding wildlife is largely understudied [1]. Such is the case of PRRSV2, which is widespread and infects domestic pigs, as well as feral pigs and wild boars [16,43–45], but the role of other wild and domestic mammals associated with farm buildings in PRRSV2 transmission is unknown [46–48]. Our

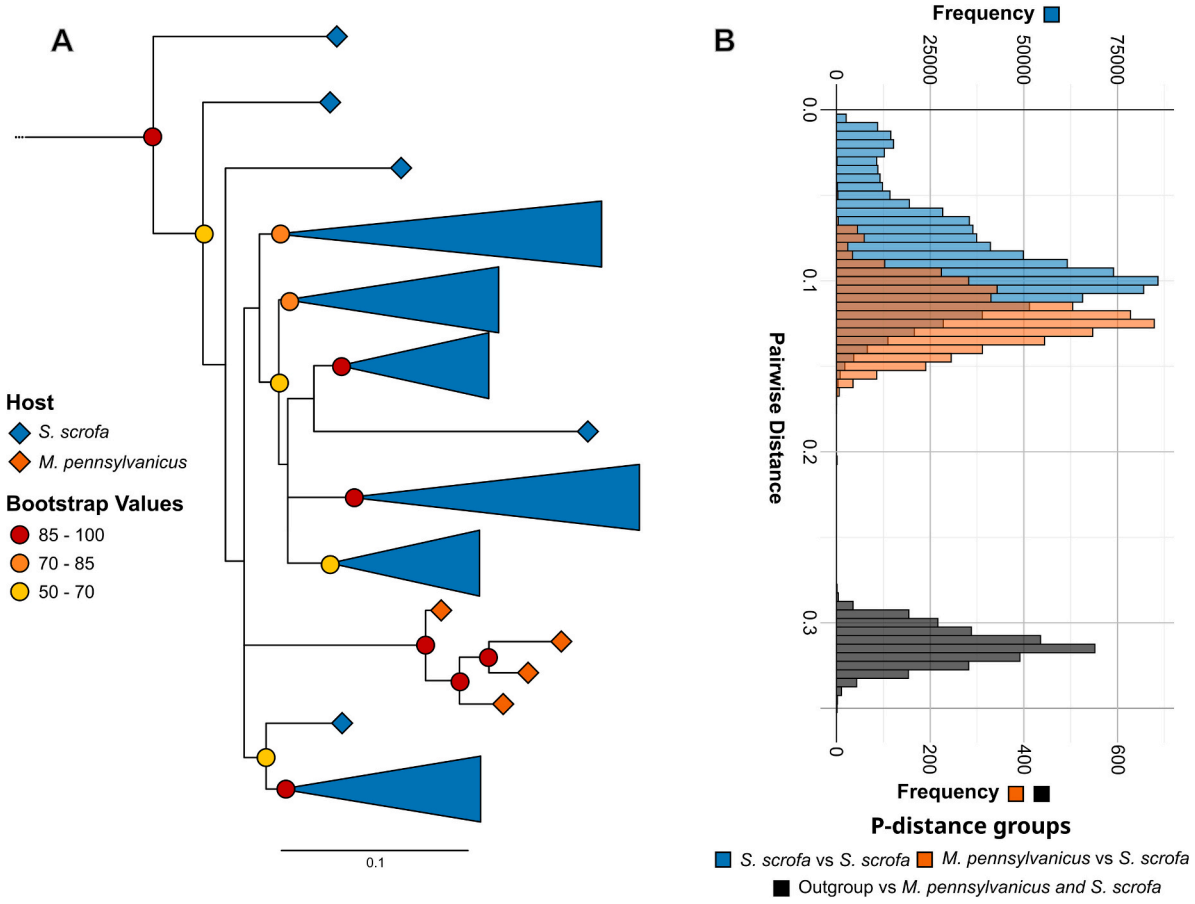

Fig. 2. Phylogenetic relationships and pairwise comparisons among ORF7 PRRSV2 sequences. A) phylogenetic tree with collapsed monophyletic nodes (see Supplementary Tree 3 for uncollapsed tree) of published *Sus scrofa* and newly obtained *Microtus pennsylvanicus* PRRSV2 sequences rooted with *Betaarterivirus sheoin* and *Betaaterivirus chinrav*. Bootstrap values are shown except when lower than 50. B) Distributions of pairwise (p-) distances (using bins of 0.005) between *S. scrofa*, *M. pennsylvanicus* and outgroup (*B. sheoin* and *B. chinrav*) PRRSV2 sequences.

results strongly suggest that PRRSV2 was present in the surveyed population of the meadow vole.

The pairwise distances calculated between the *M. pennsylvanicus* and *S. scrofa* PRRSV2 ORF7 sequences overlapped with those for the sequences within the *S. scrofa* PRRSV2 clade (Fig. 2B), supporting the conclusion that the *M. pennsylvanicus* sequences should be classified as PRRSV2. The lack of phylogenetic separation of the PRRSV2 sequences for *M. pennsylvanicus* and those for *S. scrofa* further supports that contention (Fig. 2A).

The relatively low node support for the backbone of the phylogeny in Fig. 2A means we cannot distinguish whether the *M. pennsylvanicus* clade is a nested branch in the tree (supporting a hypothesis of PRRSV2 spillover from *Sus* to *Microtus*) or whether the *M. pennsylvanicus* clade represents the closest group diverging from that of *S. scrofa* clade (supporting a *Microtus* to *Sus* spillover). In addition, Fig. S1 indicates that there is little difference in genetic distance between *S. scrofa* clusters from different geographical origins and our *M. pennsylvanicus* sequences, which does not help to resolve this issue. However, the closest relatives of PRRSV2 have been obtained from voles (here used as the outgroup), suggesting *Microtus* PRRSV2 may be the origin of the *Sus* PRRSV2 virus. This is a speculative scenario given the limited nature of our data, but possible given the wide distribution and abundance of *M. pennsylvanicus* in the northern USA [3]. In fact, the first PRRSV2 signs were noted on farms that included outdoor breeding [14]: this traditional animal husbandry practice may have allowed pigs to come into contact with vole latrines (a collection of droppings and urine) left by the rodent in the environment. It is notable that *M. pennsylvanicus* sensu lato [4,5] (but see Jackson and Cook 2019 [49]) is found in all the states in the USA where PRRSV2 was first recognized in 1989 [14] (Indiana), and where colleagues had reported very similar clinical signs in 1987 and 1988 (North Carolina, Minnesota, Iowa).

To prove that the natural reservoir of PRRSV2 is in *Microtus*, it would be important to better establish the pattern of genetic variation of PRRSV2 in voles over a wide geographic area and how it relates to the well-documented *Sus* PRRSV2 sequences. The other possibility is that the presence of PRRSV2 in meadow voles reflects transmission from wild or domestic pigs. Following a spillover event of this type, genetic diversity also tends to increase, as described for avian influenza infections in Finnish wildlife and livestock facilities [50,51]. Therefore, the presence here of relatively diverse PRRSV2 in *Microtus* compared to *Sus* sequences (Fig. S1) follows the expected pattern of farm-to-wild host spillover as well. Also, the fact that the *M. pennsylvanicus* PRRSV2 clade is nested within the *S. scrofa* PRRSV2 clade is precisely what would be expected if the pigs infected the voles. However, once again, we need to be cautious, as the backbone of the phylogeny lacks strong support. Whatever the interpretation, our results raise significant concerns about the possibility of continued PRRSV2 transmission between wild rodents and livestock and vice versa, with possibilities of new infections in pigs, suggesting the need for appropriate husbandry and surveillance measures. Controlled infection experiments could further clarify host competence and help establish whether voles serve as natural hosts; however, such experiments fall outside the scope of the present study.

As PRRSV is known to affect wild, feral and domestic pigs, it is important to consider the possible One Health consequences of the presence of this virus outside suids. Chen et al. (2025) suggest that control and prevention strategies of this virus should be based on the One Health framework, especially because PRRSV infections in pigs are known to weaken their immune system, making them more susceptible to secondary infections [52,53]. Secondary bacterial infections, such as *Streptococcus suis*, tend to be treated with antibiotics, which has resulted in an increased reliance on these compounds within the pig husbandry industry (Chen et al., 2025). In turn, this practice accelerates the rate of development of antimicrobial resistance, which is of critical public health concern [54].

Effective control of PRRSV will limit these indirect impacts on human health. The findings presented here confirm that viral metagenomics of known vector species can be a valuable tool for identifying potential virus presence and cross-species transmission, particularly from rodents to farmed animals and other economically significant species, as suggested by previous authors [18,19]. This approach should also be used to protect wildlife from the risks of farm-to-wild transmission, and prevent zoonotic viruses entering the sylvatic cycle [55,56].

5. Conclusions

This study presents the first evidence of PRRSV2 in a non-*Sus* species, the meadow vole, *M. pennsylvanicus*. Our evidence from a small sampling area of the meadow vole in New York State underscores the importance of surveying wildlife viromes to identify potential zoonotic risks, and to monitor possible sources of known disease-causing viruses. The methodology used in this study sets a potential standard for identifying circulating pathogens to understand their distribution and potential host range, which is essential for making evidence-based decisions about potential threats to human, animal, and ecosystem health. To confirm and extend our findings, there is a need for complete sequences of *M. pennsylvanicus* PRRSV2 from a wider geographic area and to investigate the presence and sequence characteristics of the virus in other North American *Microtus* (in particular, the closely related *Microtus ochrogaster* [54]). Such studies would help identify pathways of virus transmission between *Microtus* spp. and *S. scrofa*. This may help understand the origin of the PRRSV2 in pigs and the extent that voles represent a current risk to pigs. These are important issues given the continuing devastating effect of PRRSV2 on pig herds in North America and elsewhere [10,13,25].

CRediT authorship contribution statement

Elena Sgarabotto: Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Nicola Zadra:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Jacob A. Tyrell:** Writing – review & editing, Methodology, Investigation, Data curation, Conceptualization. **Chiara Rossi:** Methodology, Investigation. **Ian Hewson:** Writing – review & editing, Methodology, Investigation. **Jeremy B. Searle:** Writing – review & editing, Resources, Project administration, Investigation, Conceptualization. **Heidi C. Hauffe:** Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2026.101345.

Data availability

Quality-controlled sequence data are available via the NCBI Short Read Archive (SRA), under BioProject PRJNA1259483.

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